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BIOQUÍMICA EM SAÚDE
BIOQUÍMICA CLÍNICA E METABÓLICA

Modulation of Oxidative Stress with α-tocopherol (vitamin E) in *Saccharomyces cerevisiae*

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12/2018

Escola Superior de Saúde

Instituto Politécnico do Porto

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Dissertação submetida à Escola Superior de Saúde para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde – Ramo de Bioquímica Clínica e Metabólica, realizada sob a orientação científica da Professora Doutora Mónica Vieira, Professora Adjunta da área técnico-científica das Ciências Químicas e das Biomoléculas (ESS – P.Porto), e sob a orientação institucional da Professora Doutora Cristina Prudêncio, Professora Coordenadora com Agregação da área técnico-científica das Ciências Químicas e das Biomoléculas (ESS – P.Porto).

Dec 2018

“A human being is a part of the whole called by us universe, a part limited in time and space. He experiences himself, his thoughts and feelings as something separated from the rest, a kind of optical delusion of his consciousness. This delusion is a kind of prison for us, restricting us to our personal desires and to affection for a few persons nearest to us. Our task must be to free ourselves from this prison by widening our circle of compassion to embrace all living creatures and the whole of nature in its beauty."

Albert Einstein

Agradecimentos

À minha orientadora, Professora Doutora Mónica Vieira pela sua disponibilidade, pelos conhecimentos que me transmitiu e pelas suas palavras de incentivo, sempre.

À Sofia, pela sua disponibilidade em me receber, pela orientação no laboratório e pelos conhecimentos transmitidos.

À Dulce, por toda a ajuda, dedicação e apoio incansável, fundamentais na concretização deste trabalho.

À Rita, pelas boas conversas no edifício 8 e por me ter ajudado sempre.

À Andreia, por estar sempre lá, pelas tardes em que tanta levedura cultivámos juntas e pela boa disposição a qualquer momento do dia. À Elisabete pelas suas palavras de conforto e por todos os momentos que partilhámos ao longo destes 2 anos e meio.

Às funcionárias da faculdade, D. Elizabete e D. Lurdes, por serem umas queridas e me terem ajudado sempre.

Aos meus pais que eu amo, pelo seu apoio e incentivo.

Aos meus avós, por todo o seu carinho e sabedoria. Também lhes dedico este trabalho.

Ao João, pelos miminhos e conselhos, e a todos os meus amigos, não só pelos momentos de alegria e de união, mas também por me terem ajudado com este trabalho. São os melhores.

Ao meu Putchi, o companheiro de uma vida, por me ter aquecido o colo e o coração nos momentos penosos de escrita desta dissertação.

A todas as pessoas que de alguma forma contribuíram para tornar os meus dias mais felizes. Às crianças que me ensinam tanto e tanto carinho me dão.

A mim própria, pelas batalhas do dia-a-dia.

Abstract

Neurodegenerative diseases, such as Parkinson's Disease or Alzheimer's Disease, are characterized by the death of a subset of neurons over long periods of time. These age-related diseases are becoming more prevalent with the generalized increase of life expectancy and have been linked by many authors with increased oxidative stress levels. Indeed, oxidative stress effects can be accounted as cumulative damage, which associates well with the delayed onset and progressive nature of these conditions. Moreover, various results on life extension research strongly support the hypothesis that enhancing the cell protective systems against oxidative stress can extend life span. In view of this, the present study aimed at evaluating the role of the antioxidant α-tocopherol (vitamin E) on induced oxidative stress conditions. α-tocopherol (vitamin E) was chosen for its principal role in scavenging lipid peroxyl radicals, at lipoproteins and cell membranes, hence breaking the chain of lipid peroxidation initiated by ROS.

The toxic effect of hydrogen peroxide (H_2O_2) and the antioxidant role of vitamin E were investigated using Saccharomyces cerevisiae as a model for cell viability. A High-Performance Liquid Chromatography analysis was also performed to assess 3-nitrotyrosine and GSH:GSSG production levels, due to their relevance as oxidative stress biomarkers.

Altogether, the results presented here demonstrated that H_2O_2 exposure decreased yeast cells viability equally, independent of dose, and that the adverse effects were, at least, partially rescued by the combined exposure with vitamin E. The results from redox biomarkers were, however, shown to be inconclusive.

This preliminary study helped to understand the dual nature of vitamin E, under the conditions tested. However, future studies should be able to further explore vitamin E antioxidant role in pathological models of neurodegenerative diseases.

Key words: neurodegenerative diseases, oxidative stress, *Saccharomyces cerevisiae*, α-tocopherol, vitamin E.

Resumo

As doenças neurodegenerativas, tais como Parkinson ou Alzheimer, caracterizam-se pela morte de um conjunto de neurónios durante um longo período de tempo. Estas doenças associadas com a idade estão a tornar-se cada vez mais prevalentes na população com o aumento da esperança média de vida, e têm sido associadas, por muitos autores, com níveis aumentados de stress oxidativo. De facto, os efeitos do stress oxidativo podem ser considerados como danos cumulativos, o que se correlaciona bem com o início tardio e a natureza progressiva destas doenças. Além disso, vários resultados de estudos sobre a extensão do tempo de vida apoiam fortemente a hipótese de que o melhoramento dos sistemas de proteção celular contra o stress oxidativo pode efetivamente prolongar o tempo de vida. Neste contexto, o presente estudo teve como objetivo avaliar o papel do antioxidante α-tocoferol (vitamina E) em condições de stress oxidativo induzido. O α-tocoferol (vitamina E) foi escolhido pelo seu importante papel de remoção de radicais peroxil lipídicos, nas lipoproteínas e membranas celulares, interrompendo a cadeia de peroxidação lipídica iniciada pelas espécies reativas de oxigénio (EROs).

O efeito tóxico do peróxido de hidrogénio (H_2O_2) e o papel antioxidante da vitamina E foram investigados usando *Saccharomyces cerevisiae* como modelo para a viabilidade celular. Uma análise por Cromatografia Líquida de Alta Eficiência foi também realizada, de forma a se avaliar os níveis de produção de 3-nitrotirosina e o rácio de GSH:GSSG, dada a sua relevância enquanto biomarcadores de stress oxidativo.

Em conjunto, os resultados aqui apresentados demonstraram que a exposição ao H_2O_2 diminuiu a viabilidade das células de levedura, independentemente das concentrações testadas, e que os efeitos adversos foram, pelo menos parcialmente, recuperados pela exposição combinada com vitamina E. Os resultados dos biomarcadores de oxidação-redução foram, no entanto, inconclusivos.

Este estudo preliminar ajudou a compreender a natureza dual da vitamina E, sob as condições testadas. No entanto, estudos futuros devem ser capazes de explorar ainda mais o papel antioxidante da vitamina E em modelos patológicos de doenças neurodegenerativas.

Palavras-chave: doenças neurodegenerativas, stress oxidativo, *Saccharomyces cerevisiae*, α-tocopherol, vitamin E.

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Chapter I – Introduction

1 | Oxidative Stress

The sequential reduction of molecular oxygen through the addition of electrons leads to the formation of a number of intermediate partially reduced oxygen species, with one or more unpaired electrons, collectively termed reactive oxygen species (ROS) (Chiurchiù, Orlacchio, & Maccarrone, 2016; Riley, 2009). In homeostatic cells of aerobic organisms, the generation of ROS is well controlled by various antioxidant molecules and enzymes, with ROS performing important functions throughout the organism, from defense against external agents to intracellular and external signaling. For example, it has been reported that ROS regulate cellular growth and apoptosis, as well as blood pressure, cognitive function and immune function (Di Meo, Reed, Venditti, & Victor, 2016). However, when ROS levels exceed the antioxidant capacity of a cell, it enters a stress oxidative state. If the cell cannot revert this redox unbalance, destruction of cellular components through structural modification will occur (lipids, proteins, sugars and nucleic acids) and the cell will ultimately die (Chiurchiù et al., 2016; Coyle & Puttfarcken, 2016; Gagné, 2014; Klein & Ackerman, 2003). The main ROS molecules are presented in Table I.

Table I: Main ROS molecules, radicals and non-radicals.

Radicals		**Non-radicals**	
Formula	Name	Formula	Name
$O_2^{\bullet-}$	Superoxide	H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl	$HOCl^{-}$	Hypochlorous acid
$ROO^{\bullet}$	Peroxyl	O_3	Ozone
$RO^{\bullet}$	Alkokyl	$^1O_2$	Singlet oxygen
$HO_2^{\bullet}$	Hydroperoxyl	$ONOO^{-}$	Peroxynitrite

The generation of ROS under physiological conditions occurs as a natural by-product of aerobic metabolism. Indeed, the majority of ROS produced in cells arises from the mitochondrial respiratory chain; it is estimated that 1% to 3% of the total oxygen taken up by mitochondria during ATP production is incompletely reduced to H_2O, giving rise to the short-lived superoxide anion ($O_2^{\bullet-}$) by side reactions of complex I and III (Turrens, 1997). $O_2^{\bullet-}$ is then dismuted to H_2O_2 either spontaneously or by superoxide dismutase (SOD), an important antioxidant enzyme defense. Despite being a less potent oxidant than $O_2^{\bullet-}$, H_2O_2 can diffuse across membranes, throughout the cell, and generate the

hazardous hydroxyl radicals ($HO^{\bullet}$) in the presence of iron via the Fenton reaction (reaction 1) (Filomeni, Aquilano, & Ciriolo, 2008; Huseby, Sundkvist, & Svineng, 2008; Riley, 2009; Weissbach & Brot, 2008)**.** Hydroxyl radicals species are the most reactive of ROS and, therefore, the most damaging (Riley, 2009).

(1) $\mathbf{H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}}$

The reactivity of ROS with the signaling molecule nitric oxide ($NO^{\bullet}$) leads to the formation of many other reactive species, termed reactive nitrogen species (RNS), which also include the potent oxidant molecule peroxynitrite (ONOO−). This reactive species is produced by the combined reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ (Chiurchiù et al., 2016; Di Meo et al., 2016; Patel et al., 1999).

Alongside hydroxyl radicals, peroxynitrite is also able to cause serious damage to mithocondria, by diffusing through the its compartments and undergoing fast reactions that result in oxidation, nitration (addition of NO_2), and nitrosation (addition of NO) of critical components in the matrix, inner and outer membrane, and intermembrane space. As the respiratory chain loses efficiency, more ROS and RNS are formed causing more damage to the mitochondria. Extensive mitochondria damage leads ultimately to cell death by apoptosis (Weissbach & Brot, 2008); this is observed in different pathophysiological conditions, including main neurodegenerative diseases (Di Meo et al., 2016; Radi, Cassina, Hodara, Quijano, & Castro, 2002; Sarti et al., 2012). Indeed, the oxidising potential of $ONOO^-$ is sufficient to directly initiate lipid peroxidation reactions which results in the formation of nitrated lipids. The lipoproteins are particularly interesting targets for $ONOO^-$ as they contain a mixture of amino acids and unsaturated lipids. Hence, the reaction between RNS and fatty acids can result in the formation of both oxidised lipids as well as nitrated lipid species. Another important deleterious effect of $ONOO^-$ is the inactivation of enzymes through disruption of their iron-sulphur centres. (Patel et al., 1999). The main RNS molecules are presented in Table II.

Table II: Main RNS molecules, radicals and non-radicals

Radicals		**Non-radicals**	
Formula	Name	Formula	Name
$NO^{\bullet}$	Nitric oxide	$ONOO^{-}$	Peroxynitrite
$NO_2^{\bullet}$	Nitrogen dioxide	N_2O_3	Dinitrogen trioxide
		ONOOH	Peroxynitrous acid

Even though mitochondria is the predominant source of ROS in all cell types (Marrocco, Altieri, & Peluso, 2017), reactive species are also generated from the metabolism of xenobiotics and from enzyme systems, such NADPH oxidase, as part of the host defense mechanisms and as signalling molecules. Radiation and atmospheric pollutants are also considered exogenous sources of ROS (Huseby et al., 2008).

However, there are other sites of ROS production worth mentioning, given their importance for cellular homeostasis and association with oxidative stress under pathological conditions. Peroxisomes, the organelles that carry out oxidation reactions, generating H_2O_2, are unable to prevent the release of about 20-60% of the H_2O_2 generated inside, despite the high content of catalase (CAT) and other antioxidants. This phenomenon is believed to be enhanced under pathological conditions (Di Meo et al., 2016). Moreover, abnormalities in the endoplasmic reticulum (ER), which is involved in multiple functions, are highly associated with oxidative stress. The protein folding process (which is dependent on redox homeostasis) can get disrupted and enhance the production of misfolded proteins, consequently causing further stress (Di Meo et al., 2016; Huseby et al., 2008; Malhotra & Kaufman, 2007). In addition, the attack of free radicals to lipid membranes, especially polyunsaturated fatty acids (PUFAs), also produces lipid radicals, which are capable of oxidizing other lipids, in a chain reaction manner. This process is termed lipid peroxidation and can cause serious damage if the antioxidant systems present are not enough to scavenge the free radicals and stop the oxidative chain reaction (Ayala, Muñoz, & Argüelles, 2014; Di Meo et al., 2016). Thus, dysfunctional problems affecting these processes reportedly contribute to a greater production of reactive species which, in turn, increases cell dysfunction as well as ROS levels, in a cyclic way.

2 | Cellular defences against reactive species

Because of the potential damage that can be engendered by reactive species, aerobic organisms have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants, usually effective in neutralizing free radicals or their harmful effects, thus maintaining the redox homeostasis (Birben et al., 2012). Naturally occuring antioxidants are mainly enzymes such as superoxide dismutases (Mn-SOD in mitochondria and Cu-Zn-SOD in the cytosol), catalase (CAT), glutathione peroxidase/reductase (GPx/GR), thioredoxins, glutaredoxins and peroxiredoxin. Non-enzymatic antioxidant molecules occurring naturally are glutathione (GSH), uric acid, pyruvate or amino acids (cysteine and methionine). In addition, antioxidant molecules can also be acquired by diet, they can be lipid-soluble antioxidants, such as α-tocopherol (Vitamin E), carotenoids, quinones, as well as water-soluble antioxidants, such as ascorbic acid (Vitamin C) and some other polyphenols. These compounds act by trapping secondary oxygen radicals, such as peroxyl radicals, being themselves converted to stable free radicals in the process (Chiurchiù et al., 2016; Temple, Perrone, & Dawes, 2005). Another cytoprotective mechanism is the sequestering of transition metals ions (eg., Fe^{+2}, Cu^{+2}) in their inactive form (e.g. ferritin, ceruloplasmin) so that the cell is able to control its distribution and avoid potential damage (Di Meo et al., 2016; Repetto, Ferrarotti, & Boveris, 2010). Mechanisms have also evolved to repair and remove damaged material, which is the case of chaperones, proteasomes, lysosomes, proteases, phospholipases and DNA repair enzymes (Davies, 2016; Di Meo et al., 2016).

3 |Biological Effects of Oxidative Stress

3.1 Lipid peroxidation

Lipid peroxidation is the main molecular mechanism involved in the oxidative damage to cell structures and in the toxicity process that leads to cell death (Barrera, Pizzimenti, & Dianzani, 2008). In this process, that affects cellular membranes, lipoproteins, and other molecules containing lipids, ROS/RNS promote the abstraction of a hydrogen or the addition of an oxygen radical in unsaturated lipids, especially polyunsaturated fatty acids (PUFAs). This leads to an oxidative cascade that generates more lipid radicals and other highly reactive products (Ayala et al., 2014). Moreover, lipid peroxidation is catalysed by transition metals that not only promote free radicals by dismuting H_2O_2 into HO•, but also alter the physical properties of the bilayer when they bind to the negatively charged

phospholipids, which also favours lipid peroxidation. Considering that the central nervous system (CNS) has a high consumption of oxygen (about 20–30%), high levels of polyunsaturated fatty acids (PUFAs), and high levels of redox transition metals, it is particularly susceptible to oxidative stress (Leng et al., 2015; Repetto et al., 2010; M. Repetto, Semprine, & Boveris, 2012). The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination, which are described below (Bowry & Stocker, 1993; Josephy & Mannervik, 2006; Sachdeva, Karan, Singh, & Dhingra, 2014).

Initiation:

The lipid peroxidation is initiated, when any free radical (usually $OH^{\bullet}$) attacks and removes a hydrogen atom (H) from a fatty acid molecule (LH). This attack easily generates free radicals from PUFAs. A fatty acid radical is formed ($L^{\bullet}$):

- $\mathbf{LH + OH^{\bullet} \rightarrow L^{\bullet} + H_2O}$

Propagation phase:

In the propagation phase, the fatty acid radical ($L^{\bullet}$) takes up oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). The latter, in turn, can remove $H^{\bullet}$ from another fatty acid (LH) to form a lipid hydroperoxide (LOOH) and another $L^{\bullet}$ that continues the chain reaction.

- $\mathbf{L^{\bullet} + O_2 \rightarrow LOO^{\bullet}}$
- $\mathbf{LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}}$

In the presence of transition metal ions, the lipid hydroperoxides are capable of further stimulating lipid peroxidation, generating lipid oxyl ($LO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) radicals:

- $\mathbf{2\ LOOH \rightarrow LO^{\bullet} + LO_2^{\bullet} + H_2O}$
- **LOOH → $LO^{\bullet}$ + $LOO^{\bullet}$ + aldehydes**

Many aldehydes are produced during this phase and although highly stable when compared with free radicals, they can attack targets outside the cell, far from the site of their production, causing oxidative stress, oxidative damage and apoptosis (Repetto et al., 2012).

Termination:

Lipid peroxidation terminates when any two free radicals combine to form non-radical products. These are non-initiating and non-propagating species.

- $\mathbf{L^{\bullet} + L^{\bullet} \rightarrow LL}$
- $\mathbf{LOO^{\bullet} + L^{\bullet} \rightarrow LOOL}$
- $\mathbf{LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2}$

The chain reaction can also be broken by the action of antioxidants such as vitamin E. In vitro measurements have indicated that α-tocopherol scavenges the peroxyl radicals considerably faster than the peroxyl radical reacts with lipid substrate (Wang & Quinn, 2000). α-tocopherol is able to reduce the free radical making it insufficiently reactive to participate in further hydrogen atom abstraction reactions (Bowry & Stocker, 1993; Josephy & Mannervik, 2006; Sachdeva et al., 2014).

A prolonged oxidation of fatty acid side-chains and generation of aldehydes eventually disrupts the membrane integrity by altering its fluidity, inactivating membrane-bound proteins (e.g., sodium pump) and impairing membrane receptors (Ayala et al., 2014; Borja-Cacho & Matthews, 2008). Interestingly, there is important data linking lipid peroxidation with mitochondrial dysfunction. The mitochondrial subunits of complexes I and IV become covalently crosslinked after reaction with the lipid hydroperoxyl radicals ($LOO^{\bullet}$) and the aldehydes produced during lipid peroxidation, consequently causing protein damage. Indeed, upon aging, frontal cortex and hippocampal mitochondria show a decreased rate of respiration, with decreased enzymatic activities of complexes I and IV and increased content of oxidation products (Repetto et al., 2012).

3.2 Nucleic Acid Oxidation

ROS also affect nucleic acids causing several types of damage: bases and sugar modification, single and double strand breaks and DNA–DNA or DNA–protein cross-links (Bartesaghi & Radi, 2018; Coppedè & Migliore, 2014; Dizdaroglu, 1991; Mangialasche et al., 2009). In addition, nucleic acids can undergo nitrosative damage by RNS, which may promote nitration and deamination of purines. Therefore, oxidative/nitrosative injury to DNA and RNA can interfere with correct base pairing, impair transcriptional and post-transcriptional pathways, thus compromising protein synthesis (Mangialasche et al., 2009). Interestingly, a study has revealed a remarkable 15

fold increase in oxidized nucleotides in brain mtDNA with age. Mitochondrial respiratory chain genes are located not only on nuclear (Nu)DNA but also on mtDNA, and with a mutation rate 10 times greater than that of NuDNA and less effective repair mechanisms, the proximity of mtDNA to oxygen makes it a susceptible target for mutations. This can explain the efficiency disruption of the electron transport chain and the further oxidative stress increase with aging. Moreover, the ability of cells to respond to oxidative damage also seems to decline with age, leading to genome instability and accumulation of damaged proteins, which consequently results in altered cellular behaviour and cell death (Bartesaghi & Radi, 2018; Coyle & Puttfarcken, 2016; Mecocci, MacGarvey, & Beal, 1994). Therefore, damage to nuDNA and mtDNA may promote neuronal death through defects in oxidative phosphorylation and cell metabolism (Moreira et al., 2008)

3.3 Protein Oxidation

Protein oxidation is central in the pathogenesis and development of various neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's or ALS, and can occur directly via ROS/RNS or by secondary attack via lipid peroxidation end-products (Costa, Quintanilha, & Moradas-Ferreira, 2007). Oxidative and nitrosative stress may induce reversible or irreversible changes in proteins. Reversible changes, involved in the mechanism of redox regulation, take place mainly on the cysteine aminoacids and can be reversed by thioredoxin or glutaredoxin enzymes. Methionine is also liable to oxidation, leading to the formation of the methionine sulfoxide, repairable by the action of the methionine sulfoxide reductase (Costa et al., 2007; Pastore, Petrillo, Piermarini, & Piemonte, 2015). However, the vast majority of oxidative protein modifications are irreversible: protein fragmentation due to oxidation of the protein backbone, aggregation via covalent cross-linkages, protein carbonylation (introduction of carbonyl groups, principally ketones and aldehydes) and protein nitration (Barelli et al., 2008; Berlett & Stadtman, 1997; Mangialasche et al., 2009). Overall, these events result in intra and inter-cellular disrupted communications, which compromise cellular processes (Berlett & Stadtman, 1997; Davies, 2016). If the molecular chaperones cannot reconstitute the native structure of oxidized proteins and prevent their aggregation, the modified proteins become a target for proteolysis and degradation (Bader & Grune, 2006). However, severe oxidation causes a decrease in proteolytic susceptibility due to protein aggregation and cross-linking, also being able to inhibit proteasome activity. Therefore, failure to remove early oxidized proteins may allow for more severe modifications, generating

'indigestible' protein aggregates (Sweeney et al., 2017). These aggregates reportedly exert toxic effects and have been linked to the initiation and progression of numerous diseases (Bader & Grune, 2006; Goto & Radak, 2013). Therefore, in order to maintain cellular homeostasis and prevent the accumulation of highly oxidized and crosslinked proteins, degradation of mildly oxidized proteins is an essential function.

One hallmark of nitrosative stress is the nitration of protein tyrosine, in which the substitution of a hydrogen by a nitro group ($-NO_2$) in the position 3 of the phenolic ring, generates 3-nitrotyrosine (3-NT). The nitration mechanism comprises the reaction of nitrating species (mainly peroxynitrite) with tyrosine to form tyrosyl radicals ($Tyr^{\bullet}$), which, in turn, recombine with nitrogen dioxide (NO_2) to produce 3-nitrotyrosine (Barelli et al., 2008). Moreover, lipid radicals can also oxidize tyrosine to $Tyr^{\bullet}$ in membranes, promoting nitration reactions. Therefore, and considering that protein tyrosine nitration is consistently observed in several pathologies including neurodegenerative diseases, 3-NT has been established as a biomarker of oxidative stress in vivo, being revealed as a strong predictor of disease onset and progression (Ahsan, 2013; Bartesaghi & Radi, 2018)

4 | Impaired protein homeostasis and Neurodegeneration

Protein folding is the physical process by which a protein folds into its functional three-dimensional structure, known as the native state. This self-assembly process results from the interactions between the side chains of their constituent amino acids and is stabilized and catalysed by chaperones. However, inherited DNA mutations, random mutations, as well as oxidative damage can result in modified protein sequence, conformation and activity. If under normal physiological conditions the misfolded protein cannot be properly refolded by chaperones, systems such as the proteasome, autophagy and ER-associated degradation (ERAD) are deployed to degrade these misfolded proteins (Valastyan & Lindquist, 2014). Still, some abnormal aggregation-prone proteins are not easily recognized and degraded by the defensive proteostasis mechanisms, which can also become overloaded and unable to keep up with the misfolded proteins and aggregates (Sweeney et al., 2017; Valastyan & Lindquist, 2014). Evidently, the accumulation of misfolded proteins into aggregates is harmful to the cell, not only due to the loss of the normal protein function, but also due to the aggregates' ability to sequester other proteins or inappropriately interact with other cellular components, causing cellular stress (Stefani & Dobson, 2003). Moreover, the accumulation of one species of misfolded proteins

seems to contribute to the progressive disruption of the entire proteostatic network, which ends up affecting the folding of unrelated proteins (Gidalevitz, Ben-Zvi, Ho, Brignull, & Morimoto, 2006; Sweeney et al., 2017). Interestingly, different forms of a given disease protein have been observed during aggregation (monomers, oligomers, fibrils, mature fibrils) and evidence has emerged showing that, whereas soluble monomers or oligomers of misfolded proteins are toxic species, the insoluble, mature fibril aggregates (amyloid-like) might be cytoprotective (Swart et al., 2014; Tutar, zgur, & Tutar, 2013).

In the long term, the neurotoxic misfolded protein aggregates deposited in the central nervous system, intra and/or extracellularly, cause stress, synaptic dysfunction and ultimately neuronal loss (Sweeney et al., 2017; Tutar et al., 2013). To give just a few examples, the aggregations include intranuclear neuronal inclusions formed by various proteins with abnormally expanded polyglutamine (polyQ) tracts, such as the Htt protein in Huntington's disease (HD); intracellular inclusion bodies such as Lewy bodies (LBs) in Parkinson's disease (PD); and intracytoplasmic neurofibrillary tangles as well as extracellular amyloid deposits in Alzheimer's disease (AD) (Swart et al., 2014). The excess amounts of the pathogenic species, which accumulate with age, evoke cytotoxic mechanisms that include calcium signaling abnormalities, inhibition of proteasomal machinery, mitochondrial dysfunction leading to increased oxidative stress, ER stress and, ultimately, activation of cell death cascades (Swart et al., 2014).

Oxidative stress has been consistently pointed out as a common pathophysiological mechanism underlying various neurodegenerative disorders (Kim, Kim, Rhie, & Yoon, 2015), with various indices of ROS damage reported within the specific brain region that undergoes selective neurodegeneration, in cases of PD, AD and Amyotrophic lateral sclerosis (ALS) (Andersen, 2015). The relationship between the accumulation of misfolded disease proteins and the signs of oxidative stress is mutually exacerbating, placing oxidative stress as a cause as well as a consequence of neurodegeneration. Indeed, increased oxidative stress can not only cause proteins to misfold, it also contributes to impaired proteostasis, especially with aging. On the other hand, inherited or acquired mutations affecting certain proteins also cause oxidative stress and neurodegeneration (Andersen, 2015; Swart et al., 2014). The brain is actually particularly vulnerable to damage caused by ROS/ RNS, not only because of its high oxygen consumption and high lipid content (Floyd & Carney, 1992), but also because it is lower in antioxidant activity in comparison with other tissues, such as the liver (Uttara, Singh, Zamboni, & Mahajan,

2009). Moreover, the human brain has higher levels of iron in certain regions, which potentiates radical formation. In addition, protein quality control is also particularly challenging in neurons, not only because they have a unique cellular structure with long extensions, but most importantly, because neurons cannot dilute toxic substances by division. Therefore, neurons are highly sensitive to misfolded proteins, especially as they age (Ciechanover & Kwon, 2017), which associates well with the late onset and progression of neurodegenerative diseases (Coyle & Puttfarcken, 2016).

5 | Redox Biomarkers

Since several studies have demonstrated an increase in oxidative/nitrosative stress in patients suffering from central nervous system diseases, like AD, PD, ALS, HD and cerebro-vascular diseases (Mangialasche et al., 2009), many efforts have been made in order to identify and determine stable redox biomarkers that could reflect the biological redox status (Marrocco et al., 2017; Shi, Caudle, & Zhang, 2008). Testing for redox biomarkers in neurodegenerative disorders could offer a better insight into the development and staging of the diseases and also offer the possibility of monitoring the modulation of oxidative stress levels with antioxidant therapies (Brewer, 2011; Lloret et al., 2009; Marrocco et al., 2017) The search for biomarkers uses biochemical indices of brain dysfunction, measured in body fluids (Cerebrospinal fluid, plasma and urine) or neuroimaging techniques to monitor brain changes (Shi et al., 2008).

5.1 3-Nitrotyrosine

3-Nitrotyrosine (3-NT) is the main product of tyrosine (Tyr) oxidation and nitration, which is promoted by the peroxynitrite ($ONOO^-$) reaction with CO_2. This reaction yields secondary radicals ($CO_3^{\bullet-}$, $^{\bullet}NO_2$ and oxo-metal complexes) that will, in a two-step process, i) generate a tyrosine radical by oxidation and ii) nitrate the tyrosine radical, generating 3-NT (Fig. I). Nonetheless, 3-NT may also be generated through the catalysis of heme-containing proteins. Since most proteins contain Tyr residues and because Tyr residues are often surface-exposed (due to mildly hydrophilic character), they are also more prone to nitrosative damage. The nitration of protein Tyr residues can dramatically change protein structure, conformation and function, leading to pathological protein accumulation (Yamakura, Taka, Fujimura, & Murayama, 1998). For example, the inactivation of human MnSOD by $ONOO^-$ is caused by exclusive nitration of tyrosine 34 (Tyr34) to 3-nitrotyrosine (Yamakura et al., 1998). Indeed, $ONOO^-$ is a destructive free

radical oxidant, capable of promoting the oxidation of several lipoproteins and of nitrating Tyr residues in many proteins. However, its production is difficult to detect so usually 3-NT is used as the detectable marker for indirectly detecting $ONOO^-$ levels (Ahsan, 2013; Bartesaghi & Radi, 2018; Teixeira, Fernandes, Prudêncio, & Vieira, 2016). Increased levels of 3-NT are detected in many different diseases, including several neurodegenerative diseases (AD, PD, ALS) (Mangialasche et al., 2009; Pastore et al., 2015). Nitrotyrosine has also been linked to the degeneration of dopamine neurons, since tyrosine is the precursor of the neurotransmitter dopamine (Pastore et al., 2015).

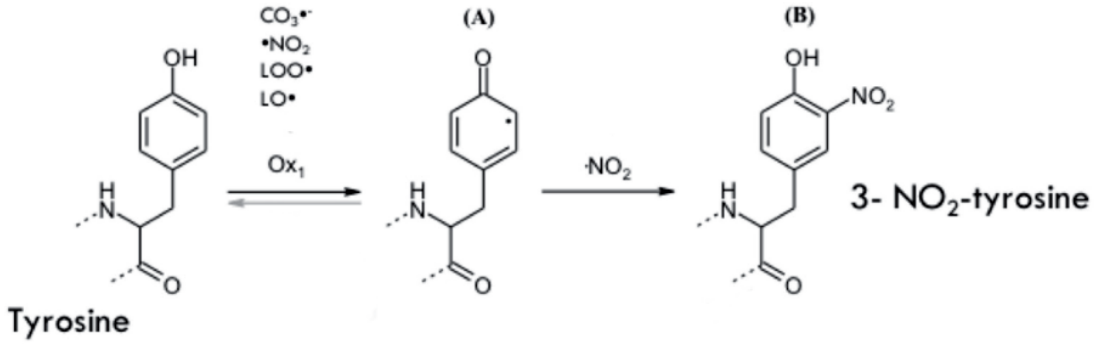

Figure I | Nitration of tyrosine to 3-nitrotyrosine. Adaptad from (Ahsan, 2013)

5.2 GSH and GSSG

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) and is the most important low molecular weight antioxidant synthesized in cells. Physiologically, GSH occurs mostly in its reduced form, which can be oxidized to GSSG under oxidative stress and is found in nearly every compartment of the cell (including the nucleus), even though 90% is stored in the cytosol, 10% in the mitochondria and a small fraction in the endoplasmic reticulum. GSH is used by the glutathione peroxidases (GPx) as an electron donor to catalyze the reduction of H_2O_2 and organic hydroperoxides to water/alcohols, thus counterbalancing the production of ROS. An impaired GPx activity has been reported in several neurodegenerative diseases. The GSH is also used by the glutathione S-transferase (GST) for the purpose of detoxification; GST catalyses the conjugation of GSH, via a sulfhydryl group, to electrophilic centers on a wide variety of substrates, increasing their water-solubility. Endogenous compounds, such as peroxidised lipids, and xenobiotics are then easier to detoxify and break down. Impaired expression of GST as well as impaired activity have also been reported in neurodegenerative diseases. The enzyme glutathione reductase (GR) is responsible for catalyzing the conversion of GSSG back to its reduced

form, GSH, using NADPH as an electron donor (Fig. II) (Forman, Zhang, & Rinna, 2010; Franco, Schoneveld, Pappa, & Panayiotidis, 2007; Pastore et al., 2015). This Gluthathione antioxidant system is the first line of antioxidant defense against ROS. Therefore, the GSH:GSSG ratio is critical and must be balanced, with GSH exceeding 100/1 the GSSG. However, under oxidative stress the ratio drops to 10/1 (Pallardó, Markovic, & Viña, 2008). A protective effect against diseases or toxic insults, in vivo or in vitro, with the administration of GSH has been reported in several studies, whereas GSH depletion showed worsening effects. In fact, the depletion of GSH is associated with oxidative stress in a variety of pathological conditions (Chi, Ke, Luo, Gozal, & Liu, 2007; Ghezzi & Simplicio, 2008; Mandal, Saharan, Tripathi, & Murari, 2015; Mischley et al., 2016)

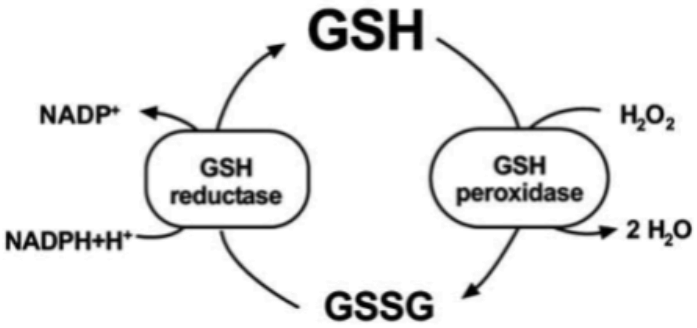

Figure II | Glutathione cycle. GSH is converted to its oxidized form, glutathione disulfide (GSSG), by the glutathione peroxidase, serving as an electron donor to catalyse the reduction of H_2O_2. Once oxidized, GSSG can be reduced back to GSH by glutathione reductase, using NADPH as an electron donor. Adapted from (Anjaneyulu, Berent-spillson, & Russell, 2008).

6 | Vitamin E

6.1 The nature of α-tocopherol (Vitamin E)

The term "vitamin E" is used to describe a group of lipophilic molecules, very similar structurally, which is composed by four tocopherols and four tocotrienols, all occurring in α, β, ϒ and δ forms. One of the most important roles of Vitamin E, an ubiquitous component of cell membranes, is its antioxidant activity. Naturally, Vitamin E can be found in fats, vegetable oils, legumes, egg yolks, nuts and seeds (Berman & Brodaty, 2004; Rizvi et al., 2014). α-tocopherol is the form of Vitamin E most abundant in nature and comprises about 90% of all tocopherols found in the tissues of animals, including humans. This is probably due to the hepatic α-tocopherol transfer protein (α-TTP), which preferentially secretes a-tocopherol, over other forms, into the plasma (Mustacich, Bruno,

& Traber, 2007). The antioxidant properties of vitamin E have been studied in the context of preventing or ameliorating the symptoms of diseases highly associated with oxidative stress, such as cardiovascular diseases, cancer and neurodegenerative disorders (Wang & Quinn, 2000). In fact, deficiency of vitamin E in humans, usually for reasons ranging from impaired absorption to genetic abnormalities in the α-TTP, may cause neurological problems, such as "cerebellar ataxia, dysarthria, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss, and positive Babinski sign" as well as immune response problems. However, administration of tocopherol can stabilize or completely reverse most of the symptoms (Brigelius-Flohé & Traber, 1999).

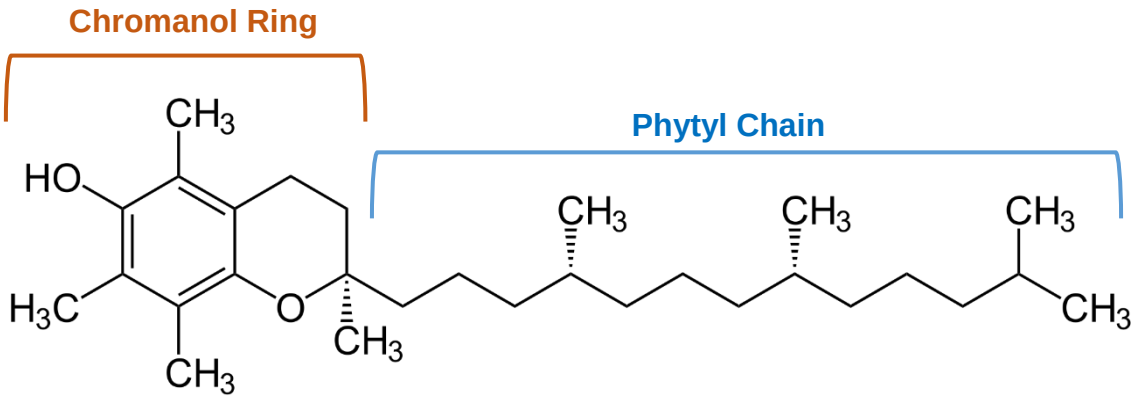

Figure III| Molecular structure of α-tocopherol (Vitamin E). Image obtained with ChemDraw JS.

The molecular structure of α-tocopherol (Fig. III) consists of a chromanol ring that is methylated at all three available positions on a benzene ring, which is attached to a 16-carbon saturated phytyl chain. The hydroxyl group (HO) on the benzene ring is responsible for the antioxidant activity. This hydrophilic group is usually near the aqueous interface, anchored to the membrane by the phytyl chain that extends towards the hydrophobic core of the membrane (Leng et al., 2015). It is known that the phytyl side chain of α-tocopherol is required for its incorporation and retention in the membranes and lipoproteins (Howard, McNeil, & McNeil, 2011; Nikis, Kawakami, Yamamoto, Tsuchiya, & Kamiya, 1985; Ulatowski & Manor, 2013).

6.2 Absorption and distribution

In humans, after solubilization by pancreatic esterases and bile acids, Vitamin E is absorbed by intestinal cells, incorporated into chylomicrons and secreted into the lymph. Once it reaches the liver, α-TTP selectively incorporates α-tocopherol into very-low-density lipoproteins (VLDLs), which are then secreted into the plasma. In the bloodstream, the VLDLs are catabolized to low-density lipoproteins (LDLs) and, in this

process, all of the circulating lipoproteins become enriched with α-tocopherol (Mustacich et al., 2007; Schmölz, Birringer, Lorkowski, & Wallert, 2016). α-tocopherol has been shown to move actively between lipoproteins of different density classes (VLDL, LDL and HDL) (Kostner et al., 1995).

The distribution of α-tocopherol, being a lipophilic molecule, tends to be associated with other lipids; allocating within lipid storage organelles and the hydrophobic domains of cell membranes. In fact, of all the subcellular membrane fractions, the greatest concentrations of α-tocopherol are found in the Golgi membranes, lysosomes and mitochondria (Wang & Quinn, 1999; Wang & Quinn, 2000) which seems to correlate well with the higher content of these membranes in PUFAs (Buttriss & Diplock, 1988).

In the CNS, the provisioning of α-tocopherol to neurons is primarily done by astrocytes. These supporting cells regulate lipid transport to neurons by synthesizing and secreting apoE-containing lipoproteins, which are critical for the redistribution of cholesterol and phospholipids in the brain (Borja-Cacho & Matthews, 2010; Huang & Mahley, 2015); indeed, apoE-/- mice have lower brain vitamin E levels (Shimmyo, Kihara, Akaike, Niidome, & Sugimoto, 2008). Also, α-TTP regulates apoE-mediated egress of α-tocopherol from the astrocytes to the neighbouring neurons. The expression of α-TTP is increased under oxidative stress conditions, suggesting an increase in vitamin delivery in order to protect neurons from oxidative stress (Ulatowski & Manor, 2013)

6.3 α-tocopherol mechanisms of action

i. Antioxidant activity

Reactive intermediates produced under conditions of oxidative stress cause the oxidation of lipids, especially of polyunsaturated fatty acids (PUFAs). α-tocopherol is the most effective lipid-soluble chain-breaking antioxidant present in biological membranes, seemingly accumulating where it is most required. Vitamin E's chromanol-head group, located within the hydrophilic portion of the bilayer, scavenges lipid peroxyl radicals by donating a hydrogen atom from an intact hydroxide group to the free radical, stabilizing it. This way, the propagation chain of free radicals is stopped and the formation of more radical species prevented (Berman & Brodaty, 2004; Howard et al., 2011; Niki, 2014; Niki & Noguchi, 2004; Phaniendra, Jestadi, & Periyasamy, 2015).

The antioxidant reaction is shown below, where LOO• represents a lipid peroxyl radical and α-TOH represents α-tocopherol. α-TOH is oxidized in the process of donating an

- **LOO• + α-TOH → LOOH + α-TOH•**

electron to LOO•, thus becoming a α-tocopheroxyloxyl radical (α-TOH•). The lipid peroxyl radical becomes a relatively stable hidroperoxide, LOOH:

The α-tocopheroxyloxyl radical formed, highly unstable, reacts either with a second lipid peroxyl radical or with a water-soluble antioxidant. The latter allows for the recycling of α-tocopherol. Each α-tocopherol molecule can donate two electrons before being consumed. The original state of α-tocopherol can be regenerated by a reduction reaction with hydroxyl groups from other antioxidants such as vitamin C (ascorbic acid) or ubiquinol (Niki & Traber, 2012; Tucker & Townsend, 2005). Interestingly, some studies found that a water-soluble vitamin E homologue, Trolox, which has the same chemical reactivity towards ROS, but limited access to the radicals within the membrane, was much less effective at inhibiting erythrocyte hemolysis induced by ROS than α-tocopherol. This highlights the importance of α-tocopherol location within the cell membranes in order to effectively break the propagation chain (Niki, 2014). Actually, studies have demonstrated that although the concentration of α-tocopherol is comparatively very low in relation to phospholipids (approximately one mole of α-tocopherol to 1,000 moles of phospholipid) (Atkinson, Epand, & Epand, 2008; Burton, Cheeseman, Doba, Ingold, & Slater, 1983), α-tocopherol clusters with microdomains enriched with PUFAs, which favours the encounter with lipid peroxyl radicals. These microdomains tend to be more curved and more fluid than other membrane regions (Ulatowski & Manor, 2013). Also, it is postulated that the chromanol group of α-tocopherol lies near the membrane surface the majority of the time, where it can easily reach water-soluble reducing agents necessary for the restoration of α-tocopherol antioxidant activity; however, the loosely packed PUFAs allows for an easy flip-flop movement of α-tocopherol within the bilayer. By bringing the chromanol group from the surface to the hydrophobic interior of the membrane, and vice versa, α-tocopherol is able to "patrol" both sides of the membrane and exert its radical scavenger activity efficiently (Leng et al., 2015).

ii. Membrane stabilizer

Vitamin E is also associated with the function of being a membrane stabilizer. The presence of vitamin E at the hydrophobic core of the plasma membrane, changes the

bilayer physical properties, such as fluidity. α-tocopherol is believed to form complexes with free fatty acids and lysophospholipids (products of membrane lipid hydrolysis), which have a tendency to destabilize the bilayer structure. The formation of these complexes result in a tighter packaging of the membrane lipids, which increases the stability of the membrane. Thereby, α-tocopherol contributes to the maintenance of membrane integrity (Rizvi et al., 2014; Wang & Quinn, 2000).

6.4 Vitamin E Depletion

Low levels of Vitamin-E in patients might be caused by low vitamin E dietary intake, genetic or absorption related problems or even as a consequence of high levels of oxidative stress. Remarkably, Vitamin E is preferentially conserved in neural tissues than in non-neural tissues; after 52 weeks of vitamin E depletion, the nervous tissues of mice still retained 5% of the normal α-tocopherol levels (Goss-Sampson, MacEvilly, & Muller, 1988), whereas other tissues were depleted of α-tocopherol after just a few weeks (Bieri, 1972). A biphasic α-tocopherol depletion is suggested for most tissues: an initial phase of rapid loss (4-8 weeks), followed by a second phase of slow depletion (Bourre & Clement, 1991). It seems plausible that the α-tocopherol mobilized in the first phase comes from the labile pool while the retained α-tocopherol should come from sub-cellular structures, where it is critical (Machlin & Gabriel, 1982). A redistribution of α-tocopherol from non-neural tissues to neural tissues during vitamin E deficiency was also observed (Muller, Lloyd, & Wolff, 1983). Interestingly, certain regions of the brain, including the forebrain and cerebellum, do not follow the biphasic pattern (Bourre & Clement, 1991); the biological half-life of vitamin E in the brain is uniquely slow, indicating tissue-specific mechanisms that regulate a CNS-specific Vitamin E pool (Muller et al., 1983).

6.5 Vitamin E in the treatment of neurological disorders

Studies on Vitamin E deficiency and supplementation have been made in order to evaluate possible beneficial effects on neurological disorders.

i. Alzheimer's disease

Studies on Alzheimer's disease have reported lower concentrations of Vitamin E in the CSF of Alzheimer's patients. In parallel with this result, an increased susceptibility of CSF to in vitro oxidation and elevated CSF markers of in vivo lipid peroxidation were also found in AD patients, indicating that oxidative stress probably has a role in the

vitamin E depletion (Kontush & Schekatolina, 2004). In a different study, supplementation with Vitamin E alone or in combination with vitamin C, significantly increased the CSF and plasma levels of vitamin E in AD patients. Also, the combined supplementation significantly decreased the susceptibility of CSF lipoproteins to *in vitro* oxidation in the presence of low amounts, but "physiologically relevant", oxidants (Kontush et al., 2001). Natural high plasma levels of vitamin E among old individuals were also found to be associated with lower risk of AD development (Mangialasche, Kivipelto, Mecocci, Rizzuto, & Palmer, 2010). Moreover, in a transgenic mice model of AD, dietary supplementation with vitamin E seemed to have beneficial effects regarding disease progression; both lipid peroxidation and amyloid-β deposition in the brain of young mice (5 months) were reduced. Supplementation at a later age (14 months), however, did not show any beneficial effects. These results put in evidence a pathological role of oxidative stress on AD early on, before amyloid deposition (Sung et al., 2004). Amyloid-β is a potent meta-chelating antioxidant (in its monomeric form) carried by lipoproteins. Therefore, the accumulation of amyloid-β peptide in the brains of AD patients can be regarded as a protective response to elevated oxidative stress. However, the increase in the amyloid-β production also increases the likelihood of aggregation and fibrillation, induced by the chelation of transition metal ions present (particularly copper). Fibrillated amyloid-β is able to produce reactive oxygen species, further aggravating oxidative stress in AD brain (Kontush, 2001; Kontush & Schekatolina, 2004). Therefore, the results suggest that treatment with antioxidants, such as vitamin E, should be helpful at an early stage of the disease or preferably, as a form of prevention (Sung et al., 2004)

However, trials with AD patients or patients suffering from mild cognitive impairment and supplementation with Vitamin E have presented controversial results. Some suggest a beneficial role for vitamin E: slower rate of progression to AD or smaller risk of developing it (Dysken et al., 2014; Kontush et al., 2001; McIntosh, Trush, & Troncoso, 1997; Morris et al., 1998, 2002; Zandi et al., 2004); interestingly, one study reported improvement on cognitive performance of subjects free of dementia that were taking vitamin E supplements (Berg et al., 2000). Some studies, however, do not find significant differences with or without vitamin E supplementation (Berg et al., 2000; Kontush & Schekatolina, 2004; Luchsinger, Tang, Shea, & Mayeux, 2003). One study even found that vitamin E supplementation can be detrimental to some AD patients (Lloret et al., 2009). Remarkably, however, most studies report beneficial results with a combined

supplementation of Vitamin E and C, on oxidative stress, AD progression or AD risk (Foley & White, 2002; Kontush et al., 2001; Morris et al., 1998; Zandi et al., 2004), which is probably due to the vitamin C capacity to regenerate α-tocopherol. Recently though, a study on the effect of one year vitamin E and C supplementation in patients with AD, revealed no beneficial effects on cognitive decline, despite the increase in vitamin concentrations in CSF and reduced in vitro oxidation of CSF lipoproteins after just one month of supplementation (Arlt, Muller-Thomsen, Beisiegel, & Kontush, 2012).

ii. Parkinson's Disease

Regarding Parkinson's Disease, decreased α-tocopherol and α-tocopherol quinone CSF levels were reported in 15 untreated PD patients compared with 14 controls (Tohgi et al., 1995). The risk of PD was shown to be significantly reduced among subjects with high intake of dietary vitamin E; however, supplementation with synthesized Vitamin E did not show beneficial results. This difference might be explained by the presence of other antioxidants in foods (Zhang, Hern, Chen, & Spiegelman, 2002). Moreover, since mitochondria oxidative stress is highly correlated with PD, one study tested the enrichment of α-tocopherol in mitochondria of mice subjected to increased concentrations of dietary α-tocopherol. The results showed a significant increase of α-tocopherol in mitochondria from liver and brain tissues of mice fed diets high in α-tocopherol. Hence, the use of chronic dietary α-tocopherol supplementation was proposed as a therapeutic strategy for the prevention or treatment of PD (Fariss & Zhang, 2003)**.**

iii. Amyotrophic Lateral Sclerosis

Studies have demonstrated an association between long-term vitamin E supplement use and lower ALS risk (H. Wang et al., 2011; Wunderink, Koster, Vries, Luyt, & Wokke, 2007) as well as slower progression (Ascherio et al., 2005; Desnuelle, Dib, Garrel, & Favier, 2001; Mustacich, Bruno, & Traber, 2007)

iv. Huntington Disease

α-tocopherol protected neuronal cells against glutamate induced cytotoxicity and attenuated cell death in a dose dependent manner (Miyamoto, Murphy, Schnaar, & Coyle, 1989). Another study revealed α-tocopherol to have beneficial effects on Huntington disease patients only when taken at an early stage of the disease. Late stage supplementation seemed to worsen the patients neurological conditions when compared to controls (Johri & Beal, 2012; Peyser et al., 1995).

6.6 The Dual Nature of Vitamin E and The Clinical Trial Controversial Results

Behind the controversial results on vitamin E supplementation, seems to be its dual nature. Vitamin E has been showed to consistently exert its antioxidant activity in strong oxidative conditions (high ROS content) (Lam, Stocker, & Dawes, 2010). But it has also been documented that on plasma lipoproteins, which are associated with only mild oxidative conditions, α-tocopherol actually acts as a pro-oxidant, by a mechanism of tocopherol-mediated peroxidation (Bowry & Stocker, 1993). Paradoxically, a computer model of the influence of Vitamin E on lipoprotein oxidation revealed that in CSF lipopoliproteins, which are also associated with mild physiological oxidative conditions, α-tocopherol acts not as a pro-oxidant, but as an antioxidant. By contrast, when these oxidative conditions are virtually increased, α-tocopherol develops antioxidant activity, both in plasma and CSF lipoproteins (Kontush & Schekatolina, 2004). Therefore, it is hypothesised that in low fluxes of oxygen radicals, the α-tocopheroxyl radicals (highly unstable) have lower probabilities of reacting with peroxyl radicals, which makes them more available to react and oxidize other PUFAs. Hence, the antioxidant activity of α-tocopherol observed in CSF lipoproteins, but not in plasma, should relate to differences in the ratios of vitamin E and oxidizable lipids, between CSF and plasma. In other words, there is different oxidative conditions per unit of vitamin E, in the presence of similar amounts of oxidants. Moreover, the presence of other water soluble electron acceptors, such as vitamin C, ubiquinol-10, GSH, NADH, cysteine or pyruvate, capable of regenerating α-tocopheroxyl radicals back to α-tocopherol, seems to be of the utmost importance for the maintenance of the antioxidant nature of Vitamin E (Bowry & Stocker, 1993; Brewer, 2011; Kontush, Finckh, Karten, Kohlschutter, & Beisiegel, 1996; Kontush et al., 2001; Kontush & Schekatolina, 2004).

Therefore, one of the reasons appointed for the controversial results on vitamin E supplementation is the toxicity associated with high doses intake, which may not be efficient in preventing chronic degenerative diseases, and may, in fact, increase mortality (Lloret et al., 2009). Notwithstanding, vitamin E does seem to have a beneficial effect on diminishing the prevalence or progression of neurodegenerative diseases when taken at an early stage, which implies a timing importance. Furthermore, the best results are obtained when vitamin E supplements are consumed along with vitamin C supplements or when associated with diets enriched with other antioxidants. This is in line with the

dual nature of vitamin E, which is dependent on the redox potential in the local cellular environment (Bowry & Stocker, 1993; Jones, 2002), Loret et al. (Lloret et al., 2009) hypothesizes that having an oxidized plasma redox potential (lower plasma GSH:GSSG ratio) can energetically block the removal of reactive species. Therefore, age, genetics, environmental and behavioural individual differences may all play a critical role on the outcome of vitamin E supplementation (Azzi, Gysin, & Kempn, 2003). In this context, Brewer, Gregory J. proposes that vitamin E supplementation therapies [for AD] should aim for a reductive shift in the metabolically oxidized redox potential (Brewer, 2011).

7 | *Saccharomyces cerevisiae* as model of study for Neurodegenerative Disorders

S. cerevisiae was the first eukaryotic organism to have its genome fully sequenced, in 1996 (Tenreiro & Outeiro, 2010). Since then, the yeast has become a biological resource widely used in the research field (Menezes, Tenreiro, Macedo, Santos, & Outeiro, 2015). A fundamental aspect for using yeast as a model of study is the conserved cellular pathways between yeast and humans, such as cell division, DNA replication, metabolism, protein folding and intracellular transport, which were first identified in yeast and then shown to be conserved in higher eukaryotes (Franssens et al., 2013; Menezes et al., 2015; Miller-fleming, Giorgini, & Outeiro, 2008; Pereira, Bessa, Soares, Leo, & Saraiva, 2012). Actually, about 30% of the genes associated with human diseases seem to have yeast orthologues. Therefore, two types of studies can be performed. If the yeast possesses an orthologue gene of a hypothetically disease-causing human gene, then studies of loss or gain of function can be made, either by deleting it or by overexpressing it. On the other hand, if the desired gene is not conserved, the human protein may be heterologously expressed in yeast cells (Oliveira, Vilaça, Santos, Costa, & Menezes, 2017). Yeast models engineered to express exogenous human risk genes for synucleinopathies (such as PD), HD and other polyglutamine (poly(Q)) diseases, AD, ALS, prion diseases and tauopathies are able to recapitulate some key cellular phenotypes (Archiv, 2010; Bharadwaj, Martins, & Macreadie, 2010; Kryndushkin & Shewmaker, 2011; Outeiro & Lindquist, 2003; Winderickx et al., 2008) and have been used to perform unbiased genetic and chemical screens for phenotypic modifiers. The yeast genome is, in fact, very well characterized, easy to manipulate genetically, and rather quick and non-expensive when compared to other eukaryotes, apart from surviving indefinitely in frozen glycerol stocks. Moreover, yeast cells have a high transformation efficiency as well as a very efficient homologous recombination pathway, which makes it very easy to insert, delete or mutate genes

(Menezes et al., 2015; Tenreiro & Outeiro, 2010). There are already numerous cDNA libraries available, as well as collections of yeast strains, and efforts are to generate a collection of strains, each harbouring a single mutation. Another advantageous feature of this eukaryotic organism is the haploid and diploid forms involved in the cell cycle, which simplifies the study of lethal mutations in heterozygous diploids and recessive mutations in haploids (Braun, Bu, Ring, Kroemer, & Madeo, 2009; Miller-fleming et al., 2008). However, the main advantage of using this simple eukaryotic model might be the possibility of largescale genetic screening studies and functional genomics, which allow for a rapid and relatively easy way of establishing gene-protein-function associations. With the huge amount of genetic and biological data being obtained, a comprehensive model of eukaryotic cell functioning is being built (Menezes et al., 2015). The emergence of next generation sequencing strategies, together with traditional genetic linkage approaches, and genome-wide association studies (GWAS), risk genes associated with human diseases are being rapidly discovered (Dhungel et al., 2015). Despite the several advantages of yeast models to study human diseases, we should not forget that several human mechanisms and biological pathways are absent in the yeast; importantly, yeast does not have a nervous system. Also, yeast fails as a model to study the multicellularity and cell–cell interactions. Therefore, all findings in yeast related to human disease must ultimately be validated in higher eukaryotes, prior to trial in humans (Miller-fleming et al., 2008). Nonetheless, yeast models of human diseases are a very useful platform for high throughput screens as a first-line of approach in the discovery of new genes that might have potential as therapeutic targets, as well as in the identification of new drugs with therapeutic value (Tenreiro, Munder, Alberti, & Outeiro, 2013).

Objectives

- Induce oxidative stress in *S.cerevisiae* cells with H_2O_2 at different concentrations;
- Explore the antioxidant nature of α-tocopherol (vitamin E) as a modulator of oxidative stress induced by H_2O_2;
- Understand the association between cell viability rates, after exposure to different oxidative conditions, and the levels of the redox biomarkers: 3-nitrotyrosine and GSH:GSSG ratio.

Chapter II – Materials and Methods

1 | Cell Viability Experiments

1.1 Yeast strain and growth conditions

i. Samples

S. cerevisiae samples grown on solid YEPD medium (Sigma-Aldrich®, Portugal) were analysed after having been incubated in YEPD broth (Sigma-Aldrich®, Portugal) for 180 min with: **1)** hydrogen peroxide (H_2O_2) at concentrations of 3 mM and 5 mM; **2)** Vitamin E (α-tocopherol acid succinate) at concentrations of 5,6 mg/L, 11,3 mg/L and 22,5 mg/L, **3)** H_2O_2 + Vitamin E (α-tocopherol acid succinate), at all concentrations.

ii. Strain

The strain used in the study was *S. cerevisiae* ATCC® 9763™ available in the laboratory of the Department of Chemical Sciences and Biomolecules of School of Health of the Polytechnic of Porto.

iii. Oxidative stress inducing agent

The agent used to induce oxidative stress was hydrogen peroxide (H_2O_2) (hydrogen peroxide 30%, Merck, New Jersey, USA).

iv. Antioxidant agent

α-tocopherol, a type of vitamin E, was used in the form of α-tocopherol acid succinate (Sigma-Aldrich®, Portugal) as the antioxidant agent. The compound was solubilized in 95% ethanol, according to (Sigma-Aldrich®) to a final concentration of 10mg/mL, which corresponds to a concentration of approximately 8 mg/mL of α-tocopherol; this solution was further filtered at 0,2 μm and stored at 4ºC protected from light.

v. Culture media and solutions

Yeast Extract Peptone Dextrose (YEPD) medium (1% yeast extract, 2% bacteriological peptone, 2% dextrose, 1,5% agar) (Sigma-Aldrich®, Portugal) was used for solid cultures, while YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) was used for suspended cultures and pre-cultures. For resuspension and dilution of the cells for sample plating, a solution of 0.85% (w/v) NaCl (Carlo Erba Reagents, Val de Reuil, France) was employed. The diluted samples were plated on YEPD agar medium (Sigma-Aldrich®, Portugal).

1.2 Pre-cultures

Active dry *Saccharomyces cerevisiae* strains were initially rehydrated in sterile distilled water at room temperature for 2h, accordingly with ATCC instructions, and grown at 30°C on YEPD agar medium for 48h hours. Fresh solid *Saccharomyces cerevisiae* cultures were then established every 48h before the start of an experiment, from a previous solid culture.

After 48h of being plated, *S. cerevisiae* was inoculated and incubated into 50mL Erlenmeyer flasks, containing 10mL of sterile YEPD medium, for approximately 16h, on an orbital shaker (130 rpm) at 30ºC. These inoculums were then used to start three independent pre-cultures, rapidly achieving an optimal optical density of 0,075-0,100 at OD_{600}nm. The pre-cultures were left to grow at 30ºC for 6h, with agitation (130 rpm). It was only after this period, during the exponential growth phase of the culture, with an OD_{600nm} between 1,500 and 1,880 (0.5 McFarland standard), that the conditions being tested were prepared. Absorbance readings were all performed on a UV-Vis spectrophotometer (GENESYS™ 20 Visible Spectrophotometer, Thermo Fisher Scientific, Wilmington, EUA).

A S.cerevisiae growth curve of CFU/mL vs incubation time, as well as a growth curve of absorbance vs incubation time were previously performed by the group of Chemical Sciences and Biomolecules of School of Health of the Polytechnic of Porto. These analyses allowed for the experimental design of this work.

1.4 Testing the effect of H_2O_2 and vitamin E on viability of yeast cultures

Each of the three independent pre-cultures, at the exponential growth phase, were equally subdivided into four Erlenmeyer flasks of 250mL: one served as the double negative control (only YEPD medium), while the others were individually exposed to a concentration of H_2O_2, Vitamin E (α-tocopherol acid succinate), and a combination of both. An additional ethanol control was also performed initially, aimed at accessing the effects of ethanol on *S. cerevisiae* viability; the volumes of EtOH tested were the same as the respective volumes used for vitamin E experiments. Yeast cultures were then incubated at 30ºC for a period of 180 min, with agitation (130rpm). Throughout this period, and at 45 min intervals, starting at minute zero (T0), 5mL and 100µL samples were taken from each condition and controls. The 5mL samples were stored in falcon

flasks at -80ºC for posterior HPLC analysis, while the 100µL samples were resuspended and serially diluted 10-fold (10^{-1}-10^{-5} CFU/mL) in NaCl solution.

1.5 CFU counting

10uL of the diluted samples were plated onto YEPD agar, using the drop plate technique (Naghili et al., 2013). Culture plates were then incubated at 30ºC for a period of 48h, after which the colony-forming units (CFU) were counted and the percentage of cell survival determined. All tests were repeated 9 times for each condition.

1.6 Statistical analysis

The data obtained from each condition (n=9) was normalized to the value of its respective double negative control at T0 time. Statistical analysis was then performed using the Two-way Analysis of Variance (*ANOVA*) test. Data on Table 1 and Graphs is presented as the mean of 6 independent measurements ± standard error of the mean (SEM). All statistical tests and graphs were performed on GraphPad Prism version 7.04 (La Jolla, CA, USA).

2 | Redox biomarkers 3-NT and GSH:GSSG quantification by HPLC

2.1 3-NT and GSH:GSSG quantification by HPLC

For 3-NT measurement, HPLC method was performed according to Teixeira et al. (Teixeira et al., 2016), while the quantification of GSH and GSSG was performed according to a previous work developed by Peixoto, V. at the Department of Chemical Sciences and Biomolecules of School of Health of the Polytechnic of Porto.

2.2 Equipment and software

All measurements were performed on a Hitachi LaChrom Elite®HPLC system (Hitachi High− Technologies Corporation, Tokyo, Japan) composed by HTA L-2130 LaChrom Elite quaternary pumps, L-2200 LaChrom Elite autosampler, L-2300 LaChrom Elite column heater and L-2455 LaChrom Elite photo DAD. EZChrom Elite Compact Software Version 3.3.2. (Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and analysis.

2.3 Mobile phase

For the quantification of 3-NT, mobile phase was prepared according to the following proportions: 0.5% CH3COOH:MeOH:H2O (15:15:70, v/v) [acetic acid (Merck S.A., Algés, Portugal); methanol (Carlo Erba Reagents, Val de Reuil, France); ultrapure water obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2 µm (Thermo Fisher Scientific, Wilmington, EUA)] and was filtered through a 0.45 µm filter membrane.

For the quantification of GSH and GSSG, mobile phase was prepared according to the following proportions: 0,085% H3PO4: H2O (1:99, v/v) [orthophosphoric acid (Merck S.A., Algés, Portugal); ultrapure water obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2 µm (Thermo Fisher Scientific, Wilmington, EUA)] and was filtered through a 0.45 µm filter membrane.

2.4 Calibration standards

Stock solutions of 3-NT (0,5 mg/L) and of GSH and GSSH combined (0,8 mg/L GSH and 4,25mg/L GSSG) were prepared using the mobile phases as solvents and filtered through a filter membrane device. Standard solutions of 0,2; 0,1; 0,05; 0,02; 0,01 and 0,005 mg/mL were prepared by diluting the stock solution with the desired mobile phase and were used for calibration purposes.

2.5 HPLC conditions

Chromatographic conditions used for 3-NT quantification were: flow rate of 1 mL/min; detection at 356 nm; volume of injection of 25 µL and oven temperature of 25°C. Chromatographic conditions used for both GSH and GSSG quantification were: flow rate of 1 mL/min; detection at 225 nm; volume of injection of 25 µL and oven temperature of 40ºC °C.

2.6 Sample preparation

The 5mL samples of *S.cerevisiae* culture collected during the experiments described above, in 45 min intervals, and for all tested conditions, were used to quantify both 3-NT and GSH:GSSG ratio.

The samples for 3-NT quantification were initially submitted twice to a cycle of freezing (10 min at -80ºC) and heating (10 min, 100ºC), aiming at the disruption of the cell wall.

Subsequently, at room temperature, glass beads (obtained manually by crushing glass to powder, in a pestle) were added to 1mL sample on round-bottom 2mL eppendorfs (1:2 w/w) and vortexed for 5min. The samples were then sonicated (Silent Crusher S at 47–63 Hz) for 10 min. After sonication, samples were centrifuged for 5 min at 14500 rpm; the obtained supernatants were centrifuged again for another 5 min at 14500 rpm and stored in a new tube at -80ºC, until appropriate assays for oxidative stress biomarkers were performed.

The samples for GSH and GSSG quantification were not submitted to freezing and heating cycles, given the poor temperature stability of these molecules. Therefore, immediately after the thawing of the samples, glass beads (obtained manually by crushing glass to powder in a pestle) were added, on ice, to 1mL sample on round-bottom 2mL eppendorfs (1:2 w/w) and vortexed for 1 min 5 times, each time keeping the cells on ice for 1 minute between vortexings. The samples were then sonicated (Silent Crusher S at 47–63 Hz) for 10 min with cold water and ice. After sonication, samples were centrifuged at 14500 rpm for 2 min 5 times, each time keeping the cells on ice for 1 minute between centrifugations. The final supernatants obtained were stored at -80ºC, until appropriate assays for oxidative stress markers were performed.

The supernatants stored were then filtered through a filter membrane device of 0.2 µm (Whatman™, Maidstone, UK) before HPLC analysis. This process was made on ice for GSH:GSSG samples. A control on temperature stability of 3-NT, GSH and GSSG was also performed for the disruption methods described.

2.7 Viability assessment

After cell wall disruption methods and before the filtering step, 10 µL of yeast cells samples were mixed with 10 µL of methylene blue 1% (PanReac AppliChem, Chicago, USA) to perform a microscopic analysis (Primo Star, ZEISS, VWR, Primo Star iLED; VisiCam 5.0: 630-1032) and assess yeast viability and cell wall integrity.

2.8 Analysed samples

One sample of each condition, as well as the respective controls, were analysed by HPLC at T0 and T180, only. Statistical analysis was not performed given the sample size. Graphs were obtained on GraphPad Prism version 7.04 (La Jolla, CA, USA).

Chapter III – Results

1 | Cell Viability Experiments

1.1 Effect of EtOH on *S. cerevisiae* viability

The results from ethanol controls did not differ significantly from their own negative controls (P>0,05), therefore validating ethanol as safe dissolving agent for acid succinate of α-tocopherol to be used in this experiment (Fig.IV).

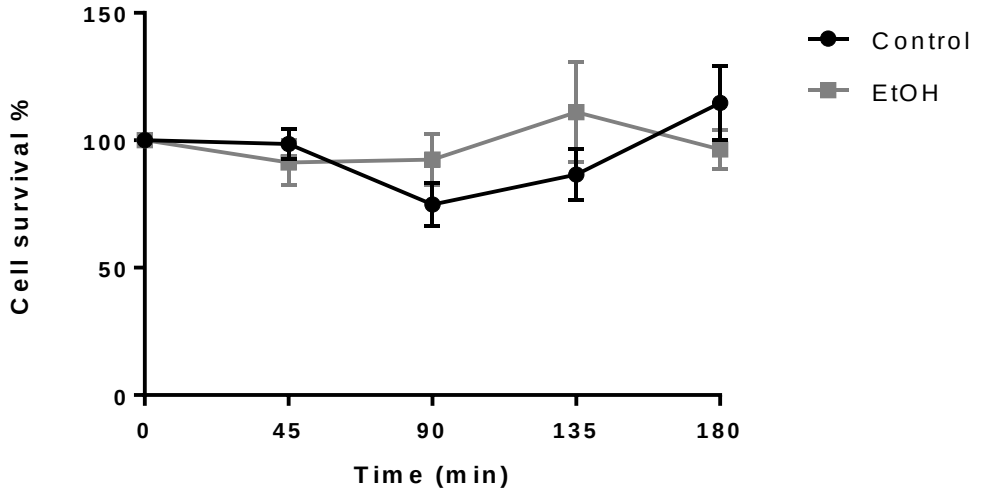

Figure IV | Effect of EtOH on *S.cerevisiae* viability. Cells were exposed to 112,5 μL of 95% EtOH for 180 min. The viability percentage was calculated as the number of c.f.u./mL obtained at each time point divided by the number of CFU/mL obtained at time 0 of the respective negative control. Data presented here represents the mean ± SEM of four independent experiments performed in triplicate (n=12).

1.2 Effect of different concentrations of H_2O_2 and vitamin E on *S.cerevisiae* Viability

Table III shows the results of the effect of different concentrations of H_2O_2 and vitamin E on *S. cerevisiae* viability. The experimental groups (G1-G11) exposed to different conditions are indicated on the table. All vitamin E-only groups (G1, G2 and G3) showed similar results to their own negative controls. Whereas in the groups where cells were exposed solely to 3 mM (G4) or 5 mM (G8) of H_20_2, it is evident an accentuated decrease in cell viability (P<0.05) of 80,6% and 79,2%, respectively.

In groups G5, G6, and G7, in which the cultured cells were exposed to H_2O_2 at 3 mM as well as to increasing concentrations of Vitamin E, we reported large differences regarding cell viability. Whereas a percentage of cell death of 81,9 and 50,0% was recorded for G5 and G7, respectively, in G6, the group exposed to an intermediate concentration of vitamin E, there was no cell death observed; in fact, a tendency of cell growth is observed.

The results from G5 did not differ significantly from G4 ($P>0.05$), showing that the lower concentration of Vitamin E is insufficient to revert cell death at these oxidative conditions. Contrastively, the results from G6 and G7 did differ significantly from G4 ($P<0.05$) indicating a protective effect of vitamin E against H_2O_2; the percentage of cell recovery was 30,6% for the higher concentration of Vitamin E and 95,7% for the intermediate concentration, which based on these results seems to completely revert the oxidant effect of H_2O_2 on cells (Table III).

In groups where the cells were exposed to 5 mM of H_20_2 and the same increasing concentrations of Vitamin E, the percentages of cell death were inversely related to the vitamin concentration, 64,4% for G9, 52,5% for G10 and 38,0% for G11 (see fig.3). Moreover, G10 and G11 results significantly differed from G8 at final recovery times (T180), but not G9, which seems to indicate once again that the lower concentration of vitamin E in unable to revert cell death significantly at these oxidative conditions. G9, G10 and G11 did not differed significantly between them.

Thus, even though the highest value on cell viability was observed for G6, the group were cells were exposed both to H_2O_2 at 3 mM and Vitamin E at 11,3mg/L, a consistent tendency on cell recovery improvement was observed for the higher concentration of H_2O_2, 5 mM. Moreover, G11 did not differ from its double negative control at final recovery time, indicating a reversal in the oxidative effect of H_2O_2.

Table III | Percentage of viability of *S. cerevisiae* cells when exposed to different concentrations of H_2O_2 and Vitamin E for 180 min. The viability percentage was calculated as the number of CFU/mL. obtained at each time point divided by the number of CFU/mL. obtained at time 0 of the respective double negative control. Data presented here represents the mean ± SEM of six independent experiments (n=6). ***** = significant difference (Two-way ANOVA $P < 0.05$) between the condition and its respective double negative control. **a, b, c, d, e =** significant difference (Two-way ANOVA $P < 0.05$) between the conditions identified with the same letter, within the same row only.

% Cell Viability					
H_2O_2	Time (min)	Vitamin E			
			5,6 mg/L	11,3 mg/L	22,5 mg/L
0	0		G1 100,0 ± 0,0	G2 100,0 ± 0,0	G3 100,0 ± 0,0
	45		69,6 ± 17,8	79,2 ± 6,3	92,0 ± 5,0
	90		87,7 ± 18,3	108,7 ± 12,6	72,9 ± 11,9
	135		74,6 ± 16,3	95,7 ± 10,1	69,8 ± 12,3
	180		90,5 ± 20,9	118,8 ± 18,4	107,4 ± 11,5
3 mM	0	G4 100,0 ± 0,0	G5 100,00 ± 0,0	G6 100,0 ± 0,0	G7 100,0 ± 0,0
	45	41,7 ± 4,2 *	56,2 ± 18,3	62,9 ± 15,3	33,6 ± 4,4 *
	90	14,3 ± 1,6 * bc	44,6 ± 13,8 d	101,0 ± 14,3 bde	42,1 ± 8,8 ce
	135	9,4 ± 1,5 * abc	33,0 ± 12,2 * d	124,7± 19,7 bde	38,4 ± 6,1 * ce
	180	19,4 ± 2,7 * bc	18,1 ± 4,0 * d	115,1 ± 14,6 * bde	50,0 ± 8,6 * ce
5 mM	0	G8 100,0 ± 0,0	G9 100,0 ± 0,0	G10 100,0 ± 0,0	G11 100,0 ± 0,0
	45	34,2 ± 3,5 * abc	77,0 ± 9,7 a	95,5 ± 9,7 b	72,0 ± 4,9 c
	90	9,0 ± 1,7 * a	52,3 ± 6,1 * a	34,9 ± 8,1 *	37,7 ± 4,2 *
	135	5,7 ± 2,0 * abc	35,3 ± 5,9 * ad	52,1 ± 9,0 b	71,6 ± 12,9 cd
	180	20,8 ± 2,7 * bc	35,6 ± 7,3 *	47,5 ± 6,2 * b	62,1 ± 18,7 c

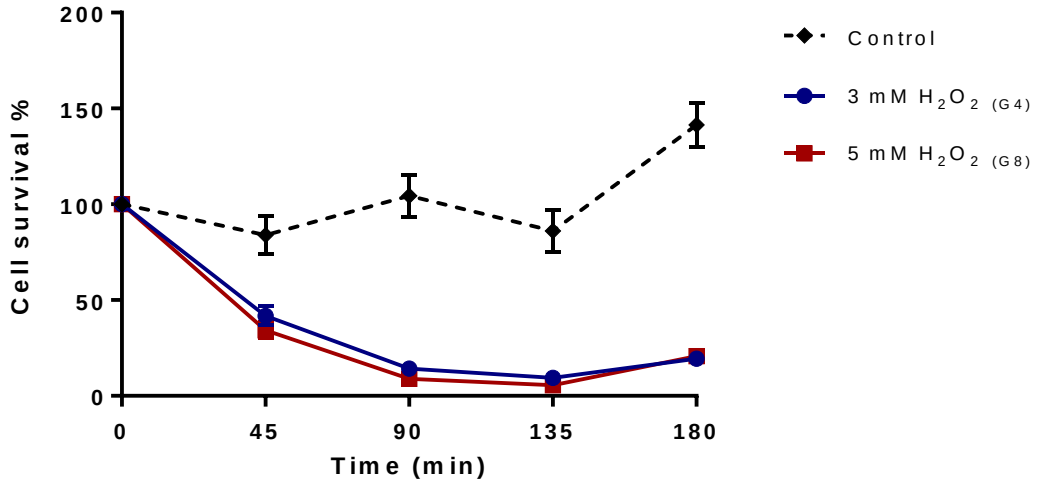

Figure V | Percentage of viability of *S. cerevisiae* when exposed to different concentrations of H_2O_2. Cells were exposed to H_2O_2 at 3 mM (G4) and 5 mM (G8), for 180 min. The viability percentage was calculated as the number of CFU/mL obtained at each time point divided by the number of CFU/mL obtained at time 0 of the respective double negative control. Data presented here represents the mean ± SEM of six independent experiments (n=6). The control shown is only indicative, since every condition group has its own double negative control group.

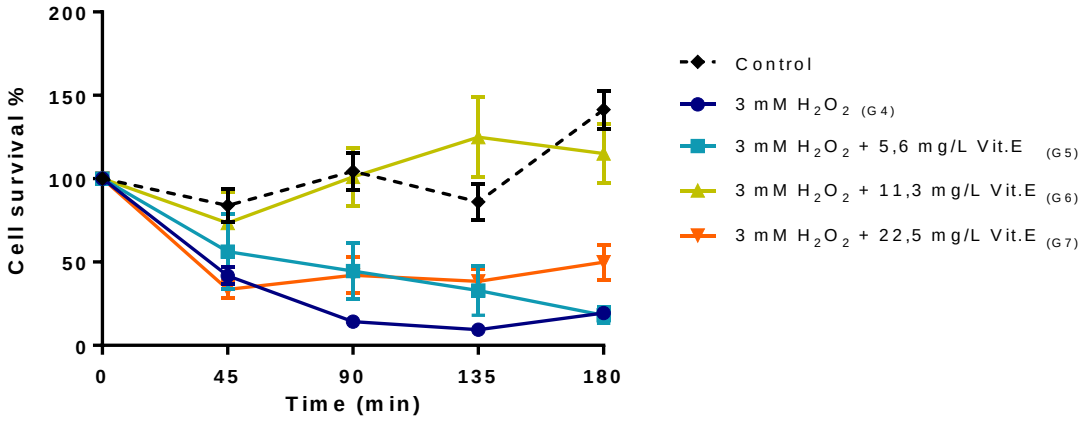

Figure VI | Percentage of viability of *S. cerevisiae* when exposed to different concentrations of Vitamin E and to 3 mM of H_2O_2. Cells were exposed to 3 mM of H_2O_2 in combination with 5,6 mg/L vitamin E (G5), 11,3 mg/L vitamin E (G6) and 22,5 mg/L vitamin E (G7) for 180 min. The results (CFU/mL) were normalized to their respective control group at T0. Data presented here represents the mean ± SEM of six independent experiments (n=6). The control shown is only indicative, since every condition group has its own double negative control group.

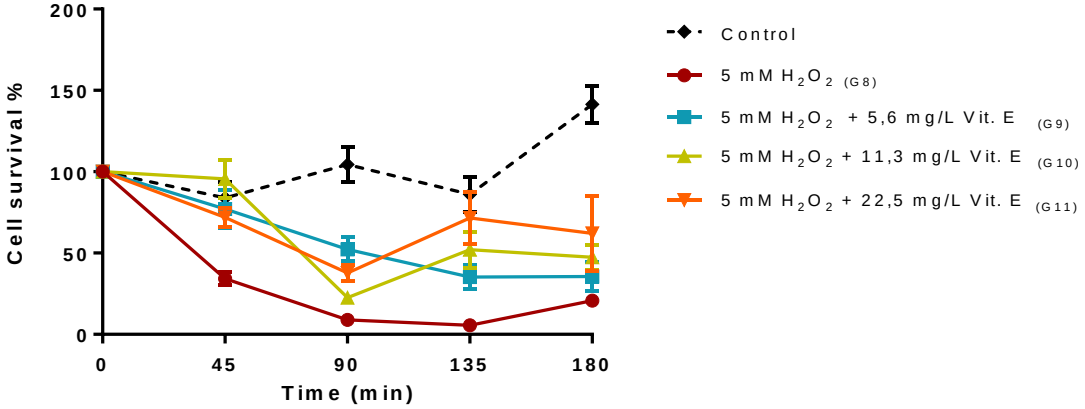

Figure VII | Percentage of viability of *S. cerevisiae when* exposed to different concentrations of Vitamin E and to 5 mM of H_2O_2. Cells were exposed to 5 mM of H_2O_2 in combination with 5,6 mg/L vitamin E (G9), 11,3 mg/L vitamin E (G10) and 22,5 mg/L vitamin E (G11) for 180 min. The results (CFU/mL) were normalized to their respective control group at T0. Data presented here represents the mean ± SEM of six independent experiments (n=6). The control shown is only indicative, since every condition group has its own double negative control group.

2. HPLC Analysis

2.1 *S. cerevisiae* cells viability

A sample of yeast cells, used as a control, was stained with methylene blue 1% and subjected to light microscopy before and after the protocol for yeast cell wall disruption with glass beads. S. cerevisiae. In a non-treated condition, yeasts were not stained, suggesting an intact cell wall, as expected (image not shown). After treatment, yeast cells were indeed stained, indicating non-viability, but continued to present its typical structure and a well-defined coloration.

Figure VIII: Light microscopy image of *S. cerevisiae* after treatment. Methylene blue staining. (Primo Star iLED, ZEISS, EUA; 40X magnification)

2.2 3-NT quantification by HPLC

After treatment for cell wall disruption, samples of each condition, as well as their respective double negative controls, at T0 and T180, were subjected to a HPLC analysis.

Samples were spiked with 3-NT and detection at 356 nm was evaluated. Results are shown on Figure IX.

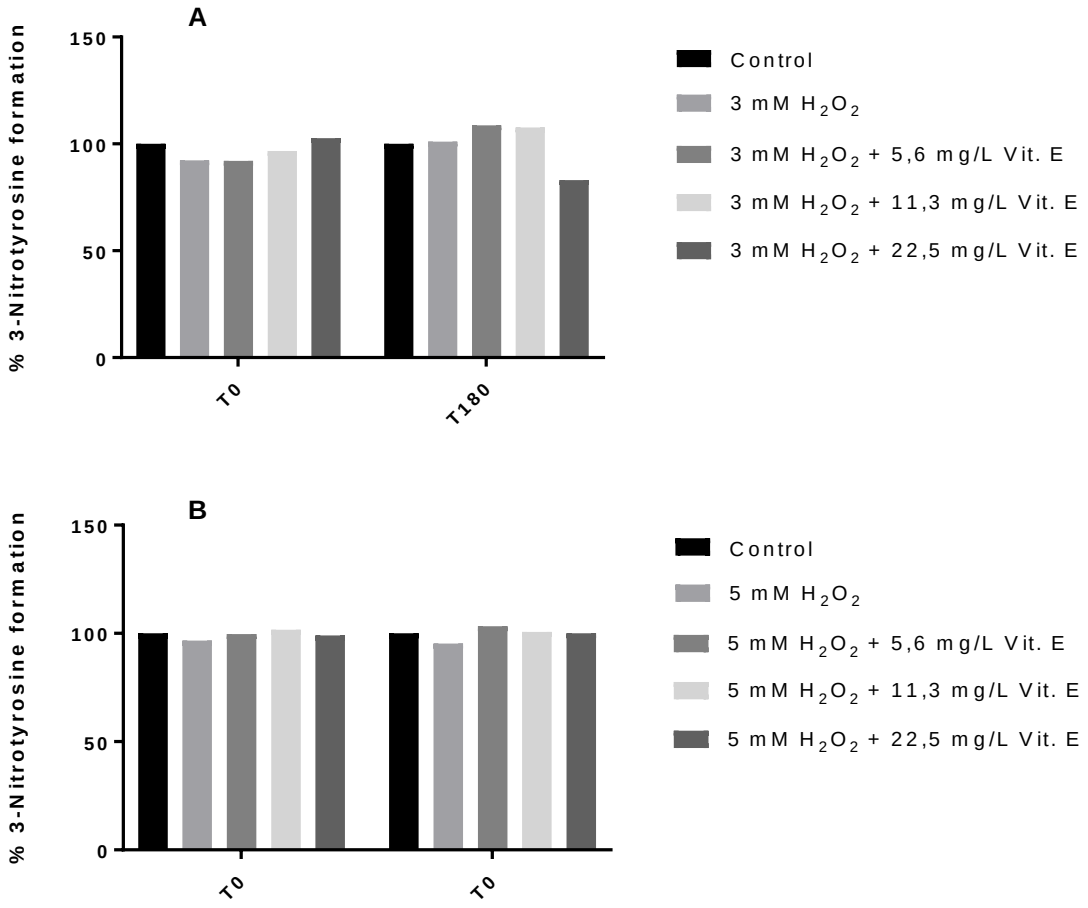

Figure IX | Data represents the percentage of 3-NT levels produced by *S. cerevisiae* exposed to different concentrations of H_2O_2 and Vitamin E, at T0 and T180. (A) Cells were exposed to 3 mM of H_2O_2 in combination with 5,6 mg/L, 11,3 mg/L and 22,5 mg/L of vitamin E, for 180 min. (B) Cells were exposed to 3 mM of H_2O_2 in combination with 5,6 mg/L, 11,3 mg/L and 22,5 mg/L of vitamin E, for 180 min. 3-NT values of exposure conditions were normalized to their respective control samples. The control shown is only indicative, since every condition has its own double negative control.

Although a statistical analysis was not possible, 3-NT levels seem to decrease at the end of the experiment (T180) for the combined concentrations of H_2O_2 at 3 mM and vitamin E at 22,5mg/L, comparatively to T0 and to control (A). However, changes on 3-NT levels at the conditions of 5 mM of H_2O_2 are not so clear; all conditions appear to have very close values of 3-NT formation (B).

2.3 GSH and GSSG quantification by HPLC

After treatment for cell wall disruption, samples of each condition, as well as their respective double negative controls, at T0 and T180, were subjected to a HPLC analysis.

Samples were spiked with GSH and GSSG and detection at 225 nm was evaluated. Results are shown on figure X.

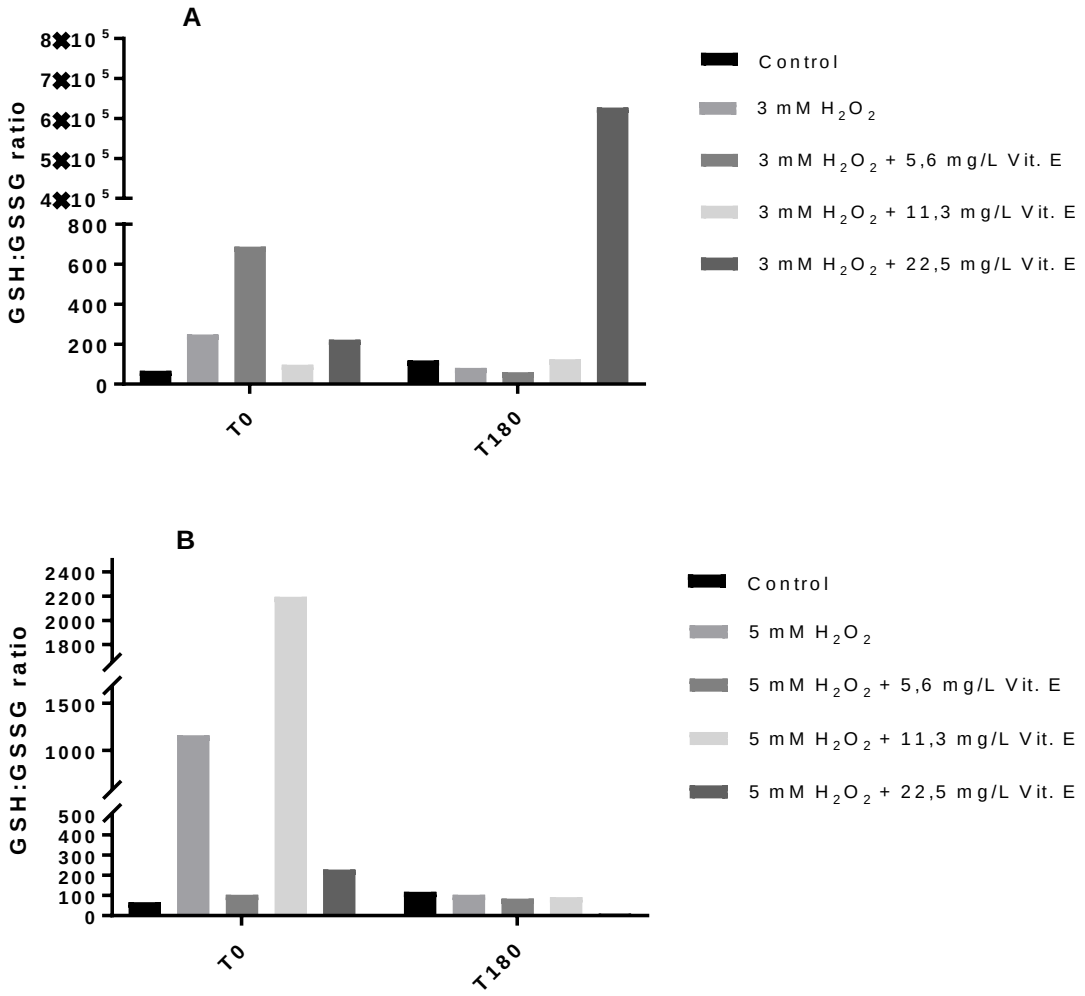

Figure X | Data represents the ratio of GSH:GSSG produced by *S.cerevisiae* exposed to different concentrations of H_2O_2 and Vitamin E, at T0 and T180. (A) Cells were exposed to 3 mM of H_2O_2 in combination with 5,6 mg/L, 11,3 mg/L and 22,5 mg/L of vitamin E, for 180 min. (B) Cells were exposed to 5 mM of H_2O_2 in combination with 5,6 mg/L, 11,3 mg/L and 22,5 mg/L of vitamin E, for 180 min. GSH and GSSG values of exposure conditions were normalized to their respective control samples. The control shown is only indicative, since every condition has its own double negative control.

Results from GSH:GSSG quantification were disregarded due to their severe inconsistency and poor sample size; values were reported with supposed differences of many orders of magnitude, compared to controls.

Chapter IV – Discussion

It has been recognized for more than fifty years that adequate levels of vitamin E are critical for maintaining neurological health (Ulatowski et al., 2014). Therefore, the use of antioxidant supplements as a form of prevention or delaying of symptoms of neurodegenerative diseases is not new. However, because their effects are not immediate and actually seem to act on a preventive manner, their therapeutic or preventive potential has been little explored. The monitoring of antioxidant levels, along with redox biomarkers on cerebrospinal fluid, could actually provide an indication of disease onset and progress; moreover, it should allow for a modulation of oxidative stress levels individually, and, consequently, more efficiently (Hensley et al., 2011). In view of this, a preliminary study was designed aimed at understanding the relationship between oxidative stress and the role of vitamin E, as an antioxidant, at normal physiological concentrations. Future studies on modulation of oxidative stress levels should focus on increasing specificity to the area of neurodegenerative diseases.

Saccharomyces cerevisiae was the eukaryotic model organism chosen for the experiments on the present study, given their easy availability and maintenance, high degree of conservation of genes and biological processes between yeast to human, short generation time and extensive literature (Franssens et al., 2013; Menezes et al., 2015; Miller-fleming et al., 2008; Pereira et al., 2012).

The present investigation studied the effect of H_2O_2 at the concentrations of 3 mM and 5 mM, which were based on previous studies using *S. cerevisiae* and H_2O_2 as an inductor of oxidative stress (Madeo et al., 1999; Pereira, Miguel Martins, & Saraiva, 2014; Salmon, Evert, Song, & Doetsch, 2004), including a previous work at the Department of Chemical Sciences and Biomolecules of School of Health of the Polytechnic of Porto.

On the other hand, to assess the antioxidant properties of vitamin E, and knowing that the reference values of α-tocopherol in plasma range from 5.5 mg/L to 17.0 mg/L (MAYO CLINIC: Mayo Medical Laboratories, s.d.), the mean value of this range was chosen as the intermediate concentration (11,25~11,3 mg/L) to be tested; double (22,5 mg/L) and half (5,6 mg/L) concentration values were tested as well, as the upper and lower limit. The objective was to assess if a lower concentration of vitamin E would have protective effects against H_2O_2 and if a higher concentration would be more or less effective. Vitamin E-only groups (G1, G2 and G3) all showed similar results to their own negative controls, which was actually not expected given that vitamin E at low oxidative conditions

is described as having a pro-oxidant nature (Bowry & Stocker, 1993; Vargas, Soares, Ribeiro, Hebling, & Costa, 2014).

Contrary to what had been planned, during data analysis, it was noticed that due to an experimental design error, the sample size was not a result of 3 individual experiments performed in triplicate, but a result of 9 individual experiments. For this reason, and given the variability of results, the closest 6 values were chosen for all statistical purposes.

H_2O_2 exposure results revealed that cells exposed to H_2O_2-only conditions presented a reduction in cell viability of 80,6% and 79,2% at the concentration of 3 mM and 5 mM, respectively. The significant cell death is in line with what has been previously observed for these H_2O_2 concentrations (Madeo et al., 1999; Magherini et al., 2007; Salmon et al., 2004), even though the results presented here show ~10% less cell death at 3 mM of H_2O_2 than previous reports (Magherini et al., 2007). Yeast cell death after 200 min of exposure to H_2O_2 at 3 mM has been explained by the accumulation of high DNA damage (massive fragmentation) which would make cell replication impossible (Magherini et al., 2007). Evaluation of cell viability with trypan blue and TUNEL assays, as well as electron microscopic investigation, were indicative of an apoptotic phenotype (Madeo et al., 1999; Magherini et al., 2007), with chromatin condensation being visible after just 30 min of H_2O_2 exposure (Madeo et al., 1999). Moreover, the concomitant decrease in the abundance of peroxiredoxin and GST (I), in a previous report, suggests that H_2O_2 treatment decreases the expression of these enzymes (or increases their degradation), leading the yeast cells to a diminished antioxidant defense, ultimately making them more prone to apoptosis (Magherini et al., 2007). The apoptotic phenotype is also described for cells incubated with 5 mM H_2O_2, while necrotic cell death seems to occur at higher concentrations (>15 mM) (Madeo et al., 1999).

On the other hand, in cells cultured with both H_2O_2 and vitamin E, it is possible to observe a tendency of cell viability recovery, which shows vitamin E antioxidant nature. At the lower concentration of H_2O_2 (3 mM), vitamin E seems to have a clear cell-protective effect, both at the intermediate (11,3mg/L) and at the highest (22,5mg/L) concentrations, with final percentages of cell viability of 115,1% (G6) and 50,0% (G7). This seems to suggest that vitamin E was able to counteract the effects of oxidative stress caused by the addition of H_2O_2 to the culture medium. In addition, since with the lower concentration of H_2O_2 and intermediate concentration of vitamin E (G6), the percentage of cell viability is higher than that of its control, it is possible that vitamin E not only prevented the toxic

effects of exogenously applied H_2O_2 but also counteracted the effects of other naturally present oxidants. Even though the viability results obtained for G6 were not observed for G7 - the group with the highest concentration of vitamin E combined with the lower concentration of H_2O_2 - an increase in cell viability, comparatively to the 3 mM H_2O_2 group (G5), was still observed ($P<0,05$). Moreover, the results from both groups with concentrations of vitamin E of 11,3 mg/L and 22,5 mg/L combined with 3 mM of H_2O_2 (G6 and G7, respectively), differed significantly from each other ($P<0,05$). This discrepancy might be explained by the oxidative role of vitamin E at relatively low oxidative conditions, which has been demonstrated in several studies (Bowry & Stocker, 1993; Kontush & Schekatolina, 2004). It might be the case that 22,5 mg/L of vitamin E is a much higher concentration than needed to counteract the toxic effects of H_2O_2 on cells at a concentration of 3 mM. It is possible that part of the vitamin added to the medium did not "find" a lipid peroxyl radical to react with and neutralize, thus initiating a new lipid peroxidation chain by reacting with a new PUFA (Bowry & Stocker, 1993). Nonetheless, in our results, the pro-oxidant action of vitamin E at relatively low oxidative conditions does not seem to be enough to aggravate cells oxidative stress, at least during the time of the experiments. In fact, the final cell viability percentage of 50,0% (G7) differed significantly from 19,4%, the final cell viability percentage of the 3 mM H_2O_2 group (G5). Results from this group (G5), however, did not differed from G6, the group with the lowest concentration of both vitamin E and H_2O_2, (except for T=135), which could indicate that the concentration of vitamin E added to the media was not enough to mitigate the effects of H_2O_2, even though a slight increase on cell viability throughout the 180 min of experiment is observable.

In regards to the 5 mM H_2O_2 groups, there seems to be a tendency towards cell viability increase proportional to the increase in vitamin E concentrations; therefore, the best results were achieved with the highest vitamin E concentration, 22,5 mg/L (G11). In fact, this group results did not differ from its own control group ($P>0,05$), indicating a reversal of oxidative stress effects. Overall, the recovery rates obtained with vitamin E at 5 mM H_2O_2 conditions were higher than those obtained for 3 mM H_2O_2, which is in line with the dual nature of α-tocopherol but does not correlate well with the absence of significant differences on cell viability between both H_2O_2 concentrations.

It is, however, not clear whether the effect of the exogenously applied H_2O_2 and of vitamin E as an antioxidant, adequately mimic the environment experienced by *S.*

cerevisiae under normal conditions, or even if it resembles in some way the environment inside an organism, as would be the case of neurons. It is hypothesized that given the slow rate of α-tocopherol movement between bilayer leaflets, and in the absence of an active uptake mechanism, exogenously applied α-tocopherol would take very long to interact with intracellular reactive species, primarily reaching the outer leaflet of cell membranes (Atkinson et al., 2008). Therefore, and because in the experiments performed for this work α-tocopherol and H_2O_2 were applied simultaneously, it could mean that α-tocopherol reacted, mainly, with exogenous ROS, preventing their toxic effects.

The 3-NT and GSH:GSSG quantification results, obtained by HPLC, were not elucidative given both the poor sample size and incomplete yeast cell wall disruption. It is also worth mentioning that the common approach for 3-NT quantification in biological samples is the previous cleavage of peptide bonds to release the free aminoacids from proteins, in tissues or fluids (Teixeira et al., 2016); usually trifluoroacetic acid (TFA) is used to promote acid hydrolysis in *S. cerevisiae*, however, TFA has a strong UV-absorption band, which can lead to HPLC baseline fluctuations that disturb high-sensitivity measurements (Choikhet, Glatz, & Rozing, 2003). Moreover, nitration of Tyr residues might occur during the application of this technique, due to the acid conditions and to the presence of nitrite in the sample (Marrocco et al., 2017). Therefore, the use of TFA was not an option during the current work. However, despite knowing that 3-NT levels are generally increased under conditions of oxidative stress, as previously mentioned, the maintenance of 3-NT levels was also expected given that Sies et al., found GPx to rapidly reduce $ONOO^-$ to nitrite, thus protecting against formation of 3-NT (Sies, Sharov, Klotz, Briviba, & Du, 1997). The results obtained with this work, particularly at the 5 mM H_2O_2 conditions, seem, nonetheless, in line with this report.

Alongside oxidative stress, an increase in GSH:GSSG ratio would be expected for both 3 mM and 5 mM of H_2O_2 conditions. Previous studies have reported a significant increase in GPx activity along with a significant increase in the ratio of GSH:GSSG in WT *S. cerevisiae* cells exposed to 5 mM H_2O_2 for 1h. The faster decrease of free [GSSG] comparatively to free [GSH], suggests that GSSG was being rapidly reduced by glutathione reductase in response to H_2O_2; in fact a difference of eightfold in [GSSG] is observed between treated and untreated conditions (Paumi, Pickin, Jarrar, Herren, & Cowley, 2012). Moreover, Madeo at el., also found GSH levels to be decreased (~20%) in WT *S. cerevisiae* incubated for 200 min with 3 mM H_2O_2 (Madeo et al., 1999), which

is in line with the GSH results for 5 mM H_2O_2 (Paumi et al., 2012). Therefore, a decrease in GSH accompanied by an accentuated decrease in GSSG, should be expected, resulting in an increased ratio of GSH:GSSG. In this context, and accounting for its antioxidant potential, the addition of vitamin E could hypothetically lead to a smaller GSH:GSSG increase. Alternatively, because α-tocopheroxyl radicals also depend on GSH for their recycling, it could be that the highest concentration of vitamin E (22,5 mg/L) would actually accentuate the decrease of [GSH].

Thereby, and although no conclusions can be drawn from the redox biomarkers results, given their inconsistency and poor sample size, the cell viability experiments demonstrated, overall, the toxic effects of H_2O_2 and the antioxidant potential of α-tocopherol, mainly under the condition of H_2O_2 at 5 mM. In general, a larger sample size and better disruption methods were needed for more reliable results and conclusions.

Chapter V – Conclusion

Our results reinforce the already described toxic effects of H_2O_2 as an oxidative stress inducer, as well as the antioxidant properties of vitamin E. For the concentration of 3 mM H_2O_2, it was found that 11,25 mg/L vitamin E was able to prevent the toxic effects of H_2O_2. As for the higher concentration of H_2O_2, 5 mM, it was found that 22,5 mg/L was the one capable of reversing oxidative stress to a greater level, with 41,3% of recovery. A tendency of increased cell viability with increasing concentrations of vitamin E is also noticeable, mostly at 5 mM H_2O_2 conditions.

Considering the detrimental effects of oxidative stress in neurodegenerative disorders, the modulation of ROS levels with the supplementation of antioxidants, such as vitamin E, could represent a treatment option to slow down neurodegeneration and reduce associated symptoms, preferentially at early disease stages or as a form of prevention.

Overall, we consider the results from this study to be preliminary and in need of much further investigation, so that more concise and reliable results can be obtained and its insights possibly extrapolated for neurodegeneration therapeutic options.

Chapter VI – Future Perspectives

In the near future we will be analysing the levels of 3-NT and GSH:GSSG of all *S. cerevisiae* samples collected during this work, in order to increase sample size reach consistency of results.

The investigation of cell viability under lower and higher concentrations of vitamin E would also be interesting in order to comprehend what happens at the concentration limits and whether or not reversion can occur.

An interesting approach would also be to re-do these experiments with both vitamin E and vitamin C, given the beneficial results on oxidative stress reported on clinical trial patients and in vitro experiments.

In the future, we also aim to test vitamin E antioxidant potential using a neurodegenerative disease yeast model to study and compare the cellular response between a normal situation and a pathological one.

In view of this, it is also our goal to assess the influence of oxidative stress on neurotransmitters, such as GABA and glutamate, as well as to attempt modulation of neurotransmitters levels with vitamin E.

References

Ahsan, H. (2013). 3-Nitrotyrosine: A biomarker of nitrogen free radical species modified proteins in systemic autoimmunogenic conditions. *Human Immunology*, *74*(10), 1392–1399. https://doi.org/10.1016/j.humimm.2013.06.009

Andersen, J. (2015). Oxidative stress in neurodegeneration: Cause or consequence? *Nature*, (February). https://doi.org/10.1038/nrn1434

Anjaneyulu, M., Berent-spillson, A., & Russell, J. W. (2008). Metabotropic Glutamate Receptors (mGluRs) and Diabetic Neuropathy. *Current Drug Targets*, 85–93.

Archiv, G. (2010). Modelling neurodegeneration in Saccharomyces cerevisiae : why cook with baker ' s yeast ? *Nature*, *11*(6), 436–449. https://doi.org/10.1038/nrn2809

Arlt, S., Muller-Thomsen, T., Beisiegel, U., & Kontush, A. (2012). Effect of One-Year Vitamin C- and E-Supplementation on Cerebrospinal Fluid Oxidation Parameters and Clinical Course in Alzheimer's Disease. *Neurochemical Research*, 2706–2714. https://doi.org/10.1007/s11064-012-0860-8

Ascherio, A., Weisskopf, M. G., Reilly, E. J. O., Jacobs, E. J., Mccullough, M. L., Calle, E. E., … Ascertainment, C. (2005). Vitamin E Intake and Risk of Amyotrophic Lateral Sclerosis. *Ann Neurol*, 1–3. https://doi.org/10.1002/ana.20316

Atkinson, J., Epand, R. F., & Epand, R. M. (2008). Tocopherols and tocotrienols in membranes : A critical review. *Free Radical Biology & Medicine*, *44*, 739–764. https://doi.org/10.1016/j.freeradbiomed.2007.11.010

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid Peroxidation : Production , Metabolism , and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal, *2014*.

Azzi, A., Gysin, R., & Kempn, P. (2003). The role of a -tocopherol in preventing disease : from epidemiology to molecular events. *Molecular Aspects of Medicine 24*, *24*, 325–336. https://doi.org/10.1016/S0098-2997(03)00028-1

Bader, N., & Grune, T. (2006). Protein oxidation and proteolysis, *387*(November), 1351–1355. https://doi.org/10.1515/BC.2006.169

Barelli, S., Canellini, G., Thadikkaran, L., Crettaz, D., Quadroni, M., Tissot, J., & Lion, N. (2008). Oxidation of proteins: Basic principles and perspectives for blood proteomics, 142–157. https://doi.org/10.1002/prca.200780009

Barrera, G., Pizzimenti, S., & Dianzani, M. U. (2008). Lipid peroxidation: control of cell proliferation, cell differentiation and cell death. *Molecular Aspects of Medicine*, *29*(1–2), 1–8. https://doi.org/10.1016/j.mam.2007.09.012

Bartesaghi, S., & Radi, R. (2018). Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox Biology*, *14*(September 2017), 618–625. https://doi.org/10.1016/j.redox.2017.09.009

Berg, A. T., Arts, W., Boulloche, J., Camfield, C. S., Camfield, P., Jallon, P., … Dlugos, D. J. (2000). Association of vitamin E and C supplement use with cognitive function and dementia in elderly men, *35*.

Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and

oxidative stress. *The Journal of Biological Chemistry*, *272*(33), 20313–20316. https://doi.org/10.1074/jbc.272.33.20313

Berman, K., & Brodaty, H. (2004). Tocopherol (Vitamin E) in Alzheimer ' s disease and other neurodegenerative disorders. *CNS Drugs*, *18*(12), 807–825.

Bharadwaj, P., Martins, R., & Macreadie, I. (2010). Yeast as a model for studying Alzheimer's disease. https://doi.org/10.1111/j.1567-1364.2010.00658.x

Bieri, J. G. (1972). KINETICS OF TISSUE a-TOCOPHEROL DEPLETION AND REPLETION. *Ann. N.Y. Acad. Sci*, 181–191.

Birben, E., Murat, U., Md, S., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative Stress and Antioxidant Defense. *WAO Journal*, *5*(January), 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Borja-Cacho, D., & Matthews, J. (2008). Lipid Peroxidation Triggers Neurodegeneration: A Redox Proteomics View into the Alzheimer Disease Brain. *Nano*, *6*(9), 2166–2171. https://doi.org/10.1021/nl061786n.Core-Shell

Borja-Cacho, D., & Matthews, J. (2010). *Apolipoprotein E: from lipid to transport to neurobiology*. *Nano* (Vol. 6). https://doi.org/10.1021/nl061786n.Core-Shell

Bourre, J. M., & Clement, M. (1991). Kinetics of rat peripheral nerve, forebrain and cerebellum α-tocopherol depletion: Comparison with different organs. *Journal of Nutrition*, *121*(8), 1204–1207. https://doi.org/10.1093/jn/121.8.1204

Bowry, V. W., & Stocker, R. (1993). Tocopherol-Mediated Peroxidation. The Prooxidant Effect of Vitamin E on the Radical-Initiated Oxidation of Human Low-Density Lipoprotein. *Journal of the American Chemical Society*, (9), 129–144.

Braun, R. J., Bu, S., Ring, J., Kroemer, G., & Madeo, F. (2009). Nervous yeast : modeling neurotoxic cell death. *Trends in Biochemical Sciences*, (November), 135–143. https://doi.org/10.1016/j.tibs.2009.10.005

Brewer, G. J. (2011). Why vitamin E therapy fails for treatment of Alzheimer disease. *Journal of Alzheimer's Disease*, *19*(1), 27–30. https://doi.org/10.3233/JAD-2010-1238.Why

Brigelius-Flohé, R., & Traber, M. G. (1999). Vitamin E: function and metabolism. *The FASEB Journal*, *13*(10), 1145–1155. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10385606

Burton, G. W., Cheeseman, K. H., Doba, T., Ingold, K. U., & Slater, T. F. (1983). Vitamin E as an antioxidant in vitro and in vivo. *Ciba Found.Symp.*, *101*(0300–5208 (Print)), 4–18.

Buttriss, J. L., & Diplock, A. T. (1988). The relationship between α-tocopherol and phospholipid fatty acids in rat liver subcellular membrane fractions. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *962*(1), 81–90. https://doi.org/10.1016/0005-2760(88)90098-7

Chi, L., Ke, Y., Luo, C., Gozal, D., & Liu, R. (2007). Depletion of Reduced Glutathione Enhances Motor Neuron Degeneration in vitro and in vivo. *Neuroscience*, *144*(3), 991–1003.

Chiurchiù, V., Orlacchio, A., & Maccarrone, M. (2016). Is modulation of oxidative stress an answer? the state of the art of redox therapeutic actions in neurodegenerative diseases. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/7909380

Ciechanover, A., & Kwon, Y. T. (2017). Protein quality control by molecular chaperones in neurodegeneration. *Frontiers in Neuroscience*, *11*(APR), 1–18. https://doi.org/10.3389/fnins.2017.00185

Coppedè, F., & Migliore, L. (2014). DNA damage in neurodegenerative diseases. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. https://doi.org/10.1016/j.mrfmmm.2014.11.010

Costa, V., Quintanilha, A., & Moradas-Ferreira, P. (2007). Protein oxidation, repair mechanisms and proteolysis in Saccharomyces cerevisiae. *IUBMB Life*, *59*(4–5), 293–298. https://doi.org/10.1080/15216540701225958

Coyle, J. T., & Puttfarcken, P. (2016). Oxidative Stress , Glutamate , and Neurodegenerative Disorders. *Science*, *262*(5134), 689–695.

Davies, M. J. (2016). Protein oxidation and peroxidation. *Biochemical Journal*, *473*(7), 805–825. https://doi.org/10.1042/BJ20151227

Desnuelle, C., Dib, M., Garrel, C., & Favier, A. (2001). A double-blind, placebo-controlled randomized clinical trial of α-tocopherol (vitamin E) in the treatment of ALS. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, *2*(1), 9–18. https://doi.org/10.1080/146608201300079364

Dhungel, N., Eleuteri, S., Masliah, E., Gitler, A. D., Dhungel, N., Eleuteri, S., … Spencer, B. (2015). Parkinson ' s Disease Genes VPS35 and EIF4G1 Interact Genetically and Converge on a -Synuclein Article Parkinson ' s Disease Genes VPS35 and EIF4G1 Interact Genetically and Converge on a -Synuclein. *Neuron*, *85*(1), 76–87. https://doi.org/10.1016/j.neuron.2014.11.027

Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. (2016). Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/1245049

Dizdaroglu, M. (1991). CHEMICAL DETERMINATION OF FREE RADICAL-INDUCED DAMAGE TO DNA. *Free Radwal Biology & Medicine*, *10*, 225–242.

Dysken, M. W., Sano, M., Asthana, S., Vertrees, J. E., Pallaki, M., Llorente, M., … Guarino, P. D. (2014). Effect of vitamin E and memantine on functional decline in Alzheimer disease: The TEAM-AD VA cooperative randomized trial. *JAMA - Journal of the American Medical Association*, *311*(1), 33–44. https://doi.org/10.1001/jama.2013.282834

Fariss, M. W., & Zhang, J. (2003). Vitamin E therapy in Parkinson ' s disease. *Toxicology*, *189*. https://doi.org/10.1016/S0300-483X(03)00158-6

Filomeni, G., Aquilano, K., & Ciriolo, M. R. (2008). GSH, Sulfur Aminoacids, and Apoptosis. In R. Masella & G. Mazza (Eds.), *Glutathione and Sulfur Amino Acids in Human Health and Disease* (pp. 211–256). John Wiley & Sons, Inc. https://doi.org/10.1002/9780470475973.ch10

Floyd, R. A., & Carney, J. M. (1992). Free Radical Damage to Protein and DNA :

Mechanisms Involved and Relevant Observations on Brain Undergoing & dative Stress. *Annals of Neurology*.

Foley, D. J., & White, L. R. (2002). Dietary Intake of Antioxidants and Risk of Alzheimer Disease. *JAMA - Journal of the American Medical Association*, *287*(24), 3261–3263.

Fomenko, D. E., Koc, A., Agisheva, N., Jacobsen, M., Kaya, A., & Malinouski, M. (2011). Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide. *PNAS*, *108*(7). https://doi.org/10.1073/pnas.1010721108

Forman, H. J., Zhang, H., & Rinna, A. (2010). Glutathione: Overview of its protective roles, measurement, and biosynthesis Henry. *Molecular Aspects of Medicine*, *30*, 1–12. https://doi.org/10.1016/j.mam.2008.08.006.Glutathione

Franco, R., Schoneveld, O. J., Pappa, A., & Panayiotidis, M. I. (2007). The central role of glutathione in the pathophysiology of human diseases. *Archives of Physiology and Biochemistry*, *113*(4–5), 234–258. https://doi.org/10.1080/13813450701661198

Franssens, V., Bynens, T., Brande, J. Van Den, Vandermeeren, K., Verduyckt, M., & Winderickx, J. (2013). The Benefits of Humanized Yeast Models to Study Parkinson ' s Disease. *Oxidative Medicine and Cellular Longevity*, *2013*.

Gagné, F. (2014). Oxidative Stress. *Biochemical Ecotoxicology: Principles and Methods*, 103–115. https://doi.org/10.1016/B978-0-12-411604-7.00006-4

Ghezzi, P., & Simplicio, P. D. I. (2008). Regulation of Protein Function by Glutathionylation. In R. Masella & G. Mazza (Eds.), *Glutathione and Sulfur Amino Acids in Human Health and Disease* (pp. 191–209). John Wiley & Sons, Inc. https://doi.org/10.1002/9780470475973

Gidalevitz, T., Ben-Zvi, A., Ho, K. H., Brignull, H. R., & Morimoto, R. I. (2006). Progressive Disruption of Cellular Protein Folding in Models of Polyglutamine Diseases. *Science*, *1471*(2006). https://doi.org/10.1126/science.1124514

Goss-Sampson, M. A., MacEvilly, C. J., & Muller, D. P. R. (1988). Longitudinal studies of the neurobiology of vitamin E and other antioxidant systems, and neurological function in the vitamin E deficient rat. *Journal of the Neurological Sciences*, *87*(1), 25–35. https://doi.org/10.1016/0022-510X(88)90051-2

Goto, S., & Radak, Z. (2013). Implications of oxidative damage to proteins and DNA in aging and its intervention by caloric restriction and exercise. *Journal of Sport and Health Science*, *2*(2), 75–80. https://doi.org/10.1016/j.jshs.2013.03.004

Hensley, K., Barnes, L. L., Christov, A., Tangney, C., Honer, W. G., Schneider, J. A., … Morris, M. C. (2011). Analysis of postmortem ventricular cerebrospinal fluid from patients with and without dementia indicates association of vitamin E with neuritic plaques and specific measures of cognitive performance. *Journal of Alzheimer's Disease*, *24*(4), 767–774. https://doi.org/10.3233/JAD-2011-101995.Analysis

Howard, A. C., McNeil, A. K., & McNeil, P. L. (2011). Promotion of plasma membrane repair by vitamin e. *Nature Communications*, *2*(1), 1–8.

https://doi.org/10.1038/ncomms1594

Huang, Y., & Mahley, R. W. (2015). Apolipoprotein E sets the stage: Response to Injury Triggers Neuropathology, including Alzheimer's Disease, *91*(2), 165–171. https://doi.org/10.1016/j.chemosphere.2012.12.037.Reactivity

Huseby, N., Sundkvist, E., & Svineng, G. (2008). Glutathione and Sulfur Containing Aminoacids: Antioxidant and Conjugation Activities. In R. Masella & G. Mazza (Eds.), *Glutathione and Sulfur Amino Acids in Human Health and Disease* (pp. 93–120). John Wiley & Sons, Inc. https://doi.org/10.1002/9780470475973

Johri, A., & Beal, M. F. (2012). Antioxidants in Huntington's disease. *BBA - Molecular Basis of Disease*, *1822*(5), 664–674. https://doi.org/10.1016/j.bbadis.2011.11.014

Jones, D. P. (2002). Redox Potential of GSH/GSSG Couple: Assay and Biological Significance. *Nature*, *348*(1999), 93–112.

Josephy, P. D., & Mannervik, B. (2006). Toxicology of Oxygen. In Oxford (Ed.), *Molecular Toxicology* (2nd ed., pp. 27–30). Oxford.

Kieffer, S., & Perrot, M. (1998). The H2O2 Stimulon in Saccharomyces cerevisiae. *The Journal of Biological Chemistry*, *273*(34), 22480–22489.

Klein, J. A., & Ackerman, S. L. (2003). Oxidative stress , cell cycle , and neurodegeneration. *J. Clin. Invest.*, *111*(6), 785–793. https://doi.org/10.1172/JCI200318182.While

Kontush, A. (2001). Amyloid-β: An antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. *Free Radical Biology and Medicine*, *31*(9), 1120–1131. https://doi.org/10.1016/S0891-5849(01)00688-8

Kontush, A., Finckh, B., Karten, B., Kohlschutter, A., & Beisiegel, U. (1996). Antioxidant and prooxidant activity of a-tocopherol in human plasma and low density lipoprotein. *Journal of Lipid Research*, *37*, 1436–1448.

Kontush, A., Mann, U., Arlt, S., Ujeyl, A., Luhrs, C., Muller-Thomsen, T., & Beisiegel, U. (2001). Influence of vitamin E and C supplementation on lipoprotein oxidation in patients with Alzheimer's disease. *Free Radical Biology & Medicine*, *31*(3), 345–354.

Kontush, A., & Schekatolina, S. (2004). Vitamin E in neurodegenerative disorders: Alzheimer's disease. *Annals of the New York Academy of Sciences*, *1031*, 249–262. https://doi.org/10.1196/annals.1331.025

Kostner, G. M., Oettl, K., Jauhiainen, M., Ehnholm, C., Esterbauer, H., & Dieplinger, H. (1995). Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells. *The Biochemical Journal*, *305 (Pt 2*, 659–667. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1136412&tool=pmcentrez&rendertype=abstract

Kryndushkin, D., & Shewmaker, F. (2011). Modeling ALS and FTLD proteinopathies in yeast An efficient approach for studying protein aggregation and toxicity. *Prion*, *5*(4), 250–257. https://doi.org/10.4161/pri.5.4.17229

Lam, Y. T., Stocker, R., & Dawes, I. W. (2010). The lipophilic antioxidants α-

tocopherol and coenzyme Q10reduce the replicative lifespan of Saccharomyces cerevisiae. *Free Radical Biology and Medicine*, *49*(2), 237–244. https://doi.org/10.1016/j.freeradbiomed.2010.04.008

Leng, X., Kinnun, J. J., Marquardt, D., Ghefli, M., Ku, N., Katsaras, J., … Wassall, S. R. (2015). Article a -Tocopherol Is Well Designed to Protect Polyunsaturated Phospholipids : MD Simulations. *Biophysical Journal*, *109*(October), 1608–1618. https://doi.org/10.1016/j.bpj.2015.08.032

Lloret, A., Badía, M.-C., Mora, N. J., Pallardó, F. V., Alonso, M.-D., & Viña, J. (2009). Vitamin E Paradox in Alzheimer's Disease: It Does Not Prevent Loss of Cognition and May Even Be Detrimental. *Journal of Alzheimer's Disease*, *17*, 143–149. https://doi.org/10.3233/JAD-2009-1033

Luchsinger, J. A., Tang, M.-X., Shea, S., & Mayeux, R. (2003). Antioxidant Vitamin Intake and Risk of Alzheimer Disease. *ARCH NEUROL*, *60*.

Machlin, L. J., & Gabriel, E. (1982). Kinetics of tissue alpha-tocopherol uptake and depletion following administration of high levels of vitamin E. *Annals of the New York Academy of Sciences*, *393*, 48–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6959568

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., … Main, F. (1999). Oxygen Stress : A Regulator of Apoptosis in Yeast. *The Journal of Cell Biology*, *145*(4), 757–767.

Magherini, F., Tani, C., Gamberi, T., Caselli, A., Bianchi, L., Bini, L., & Modesti, A. (2007). Protein expression profiles in Saccharomyces cerevisiae during apoptosis induced by H2O2. *Proteomics*, *7*(9), 1434–1445. https://doi.org/10.1002/pmic.200600796

Malhotra, J. D., & Kaufman, R. J. (2007). Endoplasmic Reticulum Stress and Oxidative Stress: A Vicious Cycle or a Double-Edged Sword? *Antioxidants & Redox Signaling*, *9*(12), 2277–2294. https://doi.org/10.1089/ars.2007.1782

Mandal, P. K., Saharan, S., Tripathi, M., & Murari, G. (2015). Brain Glutathione Levels - A Novel Biomarker for Mild Cognitive Impairment and Alzheimer's Disease. *Biological Psychiatry*, *78*(10), 702–710. https://doi.org/10.1016/j.biopsych.2015.04.005

Mangialasche, F., Kivipelto, M., Mecocci, P., Rizzuto, D., & Palmer, K. (2010). High Plasma Levels of Vitamin E Forms and Reduced Alzheimer's Disease Risk in Advanced Age. *Journal of Alzheimer's Disease*, *20*, 1029–1037. https://doi.org/10.3233/JAD-2010-091450

Mangialasche, F., Polidori, M. C., Monastero, R., Ercolani, S., Camarda, C., Cecchetti, R., & Mecocci, P. (2009). Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment. *Ageing Research Reviews*, *8*(January), 285–305. https://doi.org/10.1016/j.arr.2009.04.002

Marrocco, I., Altieri, F., & Peluso, I. (2017). Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2017/6501046

McIntosh, L. J., Trush, M. A., & Troncoso, J. C. (1997). Increased susceptibility of

Alzheimer's disease temporal cortex to oxygen free radical-mediated processes. *Free Radical Biology & Medicine*, *23*(2), 183–190.

Mecocci, P., MacGarvey, U., & Beal, M. F. (1994). Oxidative Damage to Mitochondrial DNA Is lncreased in Alzheimer's Disease, 747–751.

Menezes, R., Tenreiro, S., Macedo, D., Santos, C., & Outeiro, T. (2015). From the baker to the bedside: yeast models of Parkinson's disease. *Microbial Cell*, *2*(8), 262–279. https://doi.org/10.15698/mic2015.08.219

Miller-fleming, L., Giorgini, F., & Outeiro, T. F. (2008). Yeast as a model for studying human neurodegenerative disorders. *Biotechnology Journal*, 325–338. https://doi.org/10.1002/biot.200700217

Mischley, L. K., Standish, L. J., Weiss, N. S., Padowski, J. M., Kavanagh, T. J., White, C. C., & Rosenfeld, M. E. (2016). Glutathione as a Biomarker in Parkinson's Disease: Associations with Aging and Disease Severity. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/9409363

Miyamoto, M., Murphy, T. H., Schnaar, R. L., & Coyle, J. T. (1989). Antioxidants Protect Against Glutamate-Induced Neuronal Cell Line1. *J. Pharmacol. Exp. Ther.*, *250*(3), 1132–1140.

Moreira, P. I., Nunomura, A., Nakamura, M., Takeda, A., Shenk, J. C., Aliev, G., … Perry, G. (2008). Nucleic acid oxidation in Alzheimer disease. *Free Radical Biology & Medicine 44*, *44*, 1493–1505. https://doi.org/10.1016/j.freeradbiomed.2008.01.002

Morris, M. C., Beckett, L. A., Scherr, P. A., Hebert, L. E., Bennett, D. A., Field, T. S., & Evans, D. A. (1998). Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease.pdf.

Morris, M. C., Evans, D. A., Bienias, J. L., Tangney, C. C., Bennett, D. A., Wilson, R. S., & Scherr, P. A. (2002). Dietary Intake of Antioxidant Nutrients and the Risk of Incident Alzheimer Disease in a Biracial Community Study. *JAMA - Journal of the American Medical Association*, *287*(24).

Muller, D. P. ., Lloyd, J., & Wolff, O. . (1983). Vitamin E and Neurological Function. *The Lancet*, *321*(8318), 225–228. https://doi.org/10.1016/S0140-6736(83)92598-9

Mustacich, D. J., Bruno, R. S., & Traber, M. G. (2007). Vitamin E. *Vitamins and Hormones*, *76*(07), 1–21. https://doi.org/10.1016/S0083-6729(07)76001-6

Naghili, H., Tajik, H., Mardani, K., Razavi Rouhani, S. M., Ehsani, A., & Zare, P. (2013). Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. *Veterinary Research Forum : An International Quarterly Journal*, *4*(3), 179–183. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25653794%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4312378

Niki, E. (2014). Role of vitamin e as a lipid-soluble peroxyl radical scavenger: In vitro and in vivo evidence. *Free Radical Biology and Medicine*, *66*, 3–12. https://doi.org/10.1016/j.freeradbiomed.2013.03.022

Niki, E., & Noguchi, N. (2004). Dynamics of Antioxidant Action of Vitamin E. *Accounts of Chemical Research*, *37*(1), 45–51. https://doi.org/10.1021/ar030069m

Niki, E., & Traber, M. G. (2012). A history of vitamin e. *Annals of Nutrition and Metabolism*, *61*(3), 207–212. https://doi.org/10.1159/000343106

Nikis, E., Kawakami, A., Yamamoto, Y., Tsuchiya, J., & Kamiya, Y. (1985). Effect of Phytyl Side Chain of Vitamin. *THE JOURNAL OF BIOLOGICAL CHEMISTR*, 2191–2196.

Oliveira, A. V., Vilaça, R., Santos, C. N., Costa, V., & Menezes, R. (2017). Exploring the power of yeast to model aging and age-related neurodegenerative disorders. *Biogerontology*, *18*(1), 3–34. https://doi.org/10.1007/s10522-016-9666-4

Outeiro, T. F., & Lindquist, S. (2003). Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology. *Science*, *302*(5651), 1772–1775. https://doi.org/10.1126/science.1090439

Pallardó, F. V., Markovic, J., & Viña, J. (2008). Cellular Compartmentalization of Glutathione. In R. Masella & G. Mazza (Eds.), *Glutathione and Sulfur Amino Acids in Human Health and Disease* (pp. 35–45). John Wiley & Sons, Inc. https://doi.org/10.1002/9780470475973.ch3

Pastore, A., Petrillo, S., Piermarini, E., & Piemonte, F. (2015). Systemic Redox Biomarkers in Neurodegenerative Diseases. *Current Drug Metabolism*, *16*(1), 46–70. https://doi.org/10.2174/1389200216011507021612 50

Patel, R. P., McAndrew, J., Sellak, H., White, C. R., Jo, H., Freeman, B. A., & Darley-Usmar, V. M. (1999). Biological Aspects of Reactive Nitrogen Species. *Biochimica et Biophysica Acta - Bioenergetics*, *1411*(2–3), 385–400. https://doi.org/10.1016/S0005-2728(99)00028-6

Paumi, C. M., Pickin, K. A., Jarrar, R., Herren, C. K., & Cowley, S. T. (2012). Ycf1p attenuates basal level oxidative stress response in Saccharomyces cerevisiae. *FEBS Letters*, *586*(6), 847–853. https://doi.org/10.1016/j.febslet.2012.02.010

Pereira, C., Bessa, C., Soares, J., Leo, M., & Saraiva, L. (2012). Contribution of yeast models to neurodegeneration research. *Journal of Biomedicine and Biotechnology*. https://doi.org/10.1155/2012/941232

Pereira, C., Miguel Martins, L., & Saraiva, L. (2014). LRRK2, but not pathogenic mutants, protects against H2O 2 stress depending on mitochondrial function and endocytosis in a yeast model. *Biochimica et Biophysica Acta - General Subjects*, *1840*(6), 2025–2031. https://doi.org/10.1016/j.bbagen.2014.02.015

Peyser, C. E., Folstein, M., Chase, G. A., Starkstein, S., Brandt, J., Cockrell, J. R., … Folstein, S. E. (1995). Trial of d-u-Tocopherol in Huntington's Disease. *Am J Psychiatry*, (December), 1771–1775.

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian Journal of Clinical Biochemistry*, *30*(1), 11–26. https://doi.org/10.1007/s12291-014-0446-0

Radi, R., Cassina, A., Hodara, R., Quijano, C., & Castro, L. (2002). Peroxynitrite reactions and formation in mitochondria. *Free Radical Biology and Medicine*, *33*(11), 1451–1464. https://doi.org/10.1016/S0891-5849(02)01111-5

Repetto, M. G., Ferrarotti, N. F., & Boveris, A. (2010). The involvement of transition metal ions on iron-dependent lipid peroxidation. *Archives of Toxicology*, *84*(4),

255–262. https://doi.org/10.1007/s00204-009-0487-y

Repetto, M., Semprine, J., & Boveris, A. (2012). Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical Determination. In *Lipid Peroxidation* (pp. 3–30). https://doi.org/10.5772/45943

Riley, P. A. (2009). Free Radicals in Biology: Oxidative Stress and the Effects of Ionizing Radiation. *International Journal of Radiation Biology*, *65*(1), 27–33. https://doi.org/10.1080/09553009414550041

Rizvi, S., Raza, S. T., Ahmed, F., Ahmad, A., Abbas, S., & Mahdi, F. (2014). The role of Vitamin E in human health and some diseases. *Sultan Qaboos University Medical Journal*, *14*(2), 157–165.

Sachdeva, M., Karan, M., Singh, T., & Dhingra, S. (2014). Oxidants and Antioxidants in Complementary and Alternative Medicine: A Review. *Spatula DD - Peer Reviewed Journal on Complementary Medicine and Drug Discovery*, *4*(1), 1. https://doi.org/10.5455/spatula.20140131074751

Salmon, T. B., Evert, B. A., Song, B., & Doetsch, P. W. (2004). Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic Acids Research*, *32*(12), 3712–3723. https://doi.org/10.1093/nar/gkh696

Sarti, P., Forte, E., Giuffrè, A., Mastronicola, D., Magnifico, M. C., & Arese, M. (2012). The chemical interplay between nitric oxide and mitochondrial cytochrome c oxidase: Reactions, effectors and pathophysiology. *International Journal of Cell Biology*, *2012*. https://doi.org/10.1155/2012/571067

Schmölz, L., Birringer, M., Lorkowski, S., & Wallert, M. (2016). Complexity of vitamin E metabolism. *World Journal of Biological Chemistry*, *7*(1), 14. https://doi.org/10.4331/wjbc.v7.i1.14

Shi, M., Caudle, W. M., & Zhang, J. (2008). Biomarker Discovery in Neurodegenerative Diseases: A Proteomic Approach. *Neurobiology of Disease - Elsevier*, *35*(September), 1–26. https://doi.org/10.1016/j.nbd.2008.09.004.Biomarker

Shimmyo, Y., Kihara, T., Akaike, A., Niidome, T., & Sugimoto, H. (2008). Apolipoprotein E Exerts Selective and Differential Control Over Vitamin E Concentrations in Different Areas ofMammalian Brain. *Journal of Neuroscience Research*, *86*(June), 368–377. https://doi.org/10.1002/jnr

Sies, H., Sharov, V. S., Klotz, L., Briviba, K., & Du, D.-. (1997). Glutathione Peroxidase Protects against Peroxynitrite-mediated Oxidations. *The Journal of Biological Chemistry*, *272*(44), 27812–27817. https://doi.org/10.1074/jbc.272.44.27812

Stefani, M., & Dobson, C. M. (2003). Protein aggregation and aggregate toxicity : new insights into protein folding , misfolding diseases and biological evolution. *J Mol Med*, 678–699. https://doi.org/10.1007/s00109-003-0464-5

Sung, S., Yao, Y., Uryu, K., Yang, H., Lee, V. M., Trojanowski, J. Q., & Pratico, D. (2004). Early vitamin E supplementation in young but not aged mice reduces AB levels and amyloid deposition in a transgenic model of Alzheimer ' s disease, 323–

325. https://doi.org/10.1096/fj.03-0961fje

Swart, C., Haylett, W., Kinnear, C., Johnson, G., Bardien, S., & Loos, B. (2014). Neurodegenerative disorders: Dysregulation of a carefully maintained balance? *Experimental Gerontology*, *58*, 279–291. https://doi.org/10.1016/j.exger.2014.09.003

Sweeney, P., Park, H., Baumann, M., Dunlop, J., Frydman, J., Kopito, R., … Hodgson, R. (2017). Protein misfolding in neurodegenerative diseases: Implications and strategies. *Translational Neurodegeneration*, *6*(1), 1–13. https://doi.org/10.1186/s40035-017-0077-5

Teixeira, D., Fernandes, R., Prudêncio, C., & Vieira, M. (2016). 3-Nitrotyrosine quantification methods: Current concepts and future challenges. *Biochimie*. Elsevier Ltd. https://doi.org/10.1016/j.biochi.2016.02.011

Temple, M. D., Perrone, G. G., & Dawes, I. W. (2005). Complex cellular responses to reactive oxygen species. *Trends in Cell Biology*, *15*(6). https://doi.org/10.1016/j.tcb.2005.04.003

Tenreiro, S., Munder, C. M., Alberti, S., & Outeiro, T. F. (2013). Harnessing the power of yeast to unravel the molecular basis of neurodegeneration, 438–452. https://doi.org/10.1111/jnc.12271

Tenreiro, S., & Outeiro, T. F. (2010). Simple is good : yeast models of neurodegeneration. *FEMS*, *10*, 970–976. https://doi.org/10.1111/j.1567-1364.2010.00649.x

Tohgi, H., Abe, T., Saheki, M., Hamato, F., Sasaki, K., & Takahashi, S. (1995). Reduced and oxidized forms of glutathione and α-tocopherol in the cerebrospinal fluid of parkinsonian patients: comparison between before and after l-dopa treatment. *Neuroscience*, *184*, 21–24.

Tucker, J. M., & Townsend, D. M. (2005). Alpha-tocopherol : roles in prevention and therapy of human disease. *Biomedicine & Pharmacotherapy*, *59*, 380–387. https://doi.org/10.1016/j.biopha.2005.06.005

Turrens, J. F. (1997). Superoxide Production by the Mitochondrial Respiratory Chain. *Bioscience Reports*, *17*(1), 3–8.

Tutar, Y., zgur, A., & Tutar, L. (2013). Role of Protein Aggregation in Neurodegenerative Diseases. https://doi.org/10.5772/54487

Ulatowski, L. M., Parker, R., Warrier, G., Sultana, R., Butterfield, D. A., & Manor, D. (2014). Vitamin E is essential for Purkinje neuron integrity. *Neuroscience*, 120–129. https://doi.org/10.1016/j.neuroscience.2013.12.001.Vitamin

Ulatowski, L., & Manor, D. (2013). Vitamin E Trafficking in Neurologic Health and Disease. *Annual Review of Nutrition*, *33*(1), 87–103. https://doi.org/10.1146/annurev-nutr-071812-161252

Uttara, B., Singh, A. V, Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*, *7*(1), 65–74. https://doi.org/10.2174/157015909787602823

Valastyan, J. S., & Lindquist, S. (2014). Mechanisms of protein-folding diseases at a glance. *Disease Models & Mechanisms*, *7*(1), 9–14. https://doi.org/10.1242/dmm.013474

Vargas, F. D. S., Soares, D. G., Ribeiro, A. P. D., Hebling, J., & Costa, C. A. de S. C. (2014). Protective Effect of Alpha-Tocopherol Isomer from Vitamin E against the H2O2 Induced Toxicity on Dental Pulp Cells. *BioMed Research International*, *2014*, 1–5. https://doi.org/10.1155/2014/895049

Wang, H., Reilly, J. O., Weisskopf, M. G., Logroscino, G., Mccullough, M. L., Schatzkin, A., … Ascherio, A. (2011). Vitamin E Intake and Risk of Amyotrophic Lateral Sclerosis : A Pooled Analysis of Data From 5 Prospective Cohort Studies. *American Journal of Epidemiology*, *173*(6), 595–602. https://doi.org/10.1093/aje/kwq416

Wang, X., & Quinn, P. J. (1999). Vitamin E and its functions in membranes. *Progress in Lipid Research*, *38*, 309–336.

Weissbach, H., & Brot, N. (2008). Methionine Sulfoxide Reductases: A Protective System Against Oxidative Damage. In R. Masella & G. Mazza (Eds.), *Glutathione and Sulfur Amino Acids in Human Health and Disease* (pp. 157–187). John Wiley & Sons, Inc.

Winderickx, J., Delay, C., Vos, A. De, Klinger, H., Pellens, K., Vanhelmont, T., … Zabrocki, P. (2008). Protein folding diseases and neurodegeneration : Lessons learned from yeast, *1783*. https://doi.org/10.1016/j.bbamcr.2008.01.020

Wunderink, W., Koster, A., Vries, J. H. M. De, Luyt, J. Van Der, & Wokke, J. H. J. (2007). Intake of polyunsaturated fatty acids and vitamin E reduces the risk of developing amyotrophic lateral sclerosis. *JNNP*. https://doi.org/10.1136/jnnp.2005.083378

Xiaoyuan Wang, Peter J. Quinn. (2000). The location and function of vitamin E in membranes (Review). *Molecular Membrane Biology*, *17*(3), 143–156. https://doi.org/10.1080/09687680010000311

Yamakura, F., Taka, H., Fujimura, T., & Murayama, K. (1998). Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *Journal of Biological Chemistry*, *273*(23), 14085–14089. https://doi.org/10.1074/jbc.273.23.14085

Zandi, P. P., Anthony, J. C., Khachaturian, A. S., Stone, S. V, Gustafson, D., Tschanz, J. T., … Breitner, J. C. S. (2004). Reduced Risk of Alzheimer Disease in Users of Antioxidant Vitamin Supplements. *ARCH NEUROL*, *61*.